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Giovanni Marsicano

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**Physiological Role of the Cannabinoid  
Receptor 1 (CB1) in the Murine Central  
Nervous System**

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Open University

Thesis for Doctor of Philosophy in Neurobiology

Submitted: 29th January 2001

**Sponsoring Establishment: Max-Planck Institute of
Psychiatry, Munich, Germany**

Author no. R9644408
Date of submission: 1st November 2000
Date of award: 8th August 2001

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A Giulietta e Luciano Marsicano.

E a Rodolfo Sonego.

Penso che sareste contenti.

C'est pourquoi la meilleure part de notre mémoire est hors de nous, dans un souffle pluvieux, dans l'odeur de renfermé d'une chambre ou dans l'odeur d'une première flambée, partout où nous retrouvons de nous-même ce que notre intelligence, n'en ayant pas l'emploi, avait dédaigné, la dernière réserve du passé, la meilleure, celle qui, quand toutes nos larmes semblent taries, sait nous faire pleurer encore. Hors de nous? En nous pour mieux dire, mais dérobée à nos propres regards, dans un oubli plus ou moins prolongé. C'est grâce à cet oubli seul que nous pouvons de temps à autre retrouver l'être que nous fûmes, nous placer vis-à-vis des choses comme cet être l'était, souffrir à nouveau, parce que nous ne sommes plus nous, mais lui, et qu'il aimait ce qui nous est maintenant indifférent

Marcel Proust

“À l'ombre des jeunes filles en fleur”

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Abstract

The cannabinoid system is involved in many functions of mammalian brain, such as learning and memory, pain perception and locomotion. The “brain type” cannabinoid receptor CB1 is one of the key elements of the cannabinoid system. In this Thesis, some aspects of the neurobiology of mouse CB1 are described.

CB1 mRNA distribution was analysed by single and double *in situ* hybridization (ISH), revealing the expression of the receptor in specific neuronal subpopulations. This expression pattern suggests many putative functional cross-talks between the cannabinoid system and other signalling molecules in the brain, such as glutamate, GABA, cholecystokinin and nitric oxide (NO).

The putative functional interactions of the cannabinoid system with the NO pathway was studied by pharmacological treatment of neuronal NO synthase (nNOS) mutant mice with the CB1 agonist Δ^9 -tetrahydrocannabinol (Δ^9 -THC). The results showed that nNOS is necessary for some central effects of Δ^9 -THC. Moreover, ISH analysis revealed that nNOS-deficient mice contain levels of CB1 lower than normal in selected brain regions.

A “conditional” targeting approach was developed to gain insights into the specific functions of CB1 in mouse brain. By gene targeting experiments, two mutant lines were obtained. The “Flox CB1” mouse line, containing the whole open reading frame of CB1 flanked by two loxP sites will be the key tool for the generation of mouse mutants with a spatiotemporal-restricted deletion of CB1. The “CBN” mice, carrying a “null” mutation of CB1, were used for a study aimed to clarify some aspects of the *in vitro* neuroprotective activity of cannabinoids and, in particular, the involvement of CB1.

In vitro oxidative stress assays were performed on cell lines and on primary neuronal cultures derived from homozygous CBN/CBN mice and wild type littermates. The results indicate a differential protective activity of cannabinoids on cell lines and primary cultures. However, CB1 does not appear to be involved in the *in vitro* neuroprotective effects of cannabinoids.

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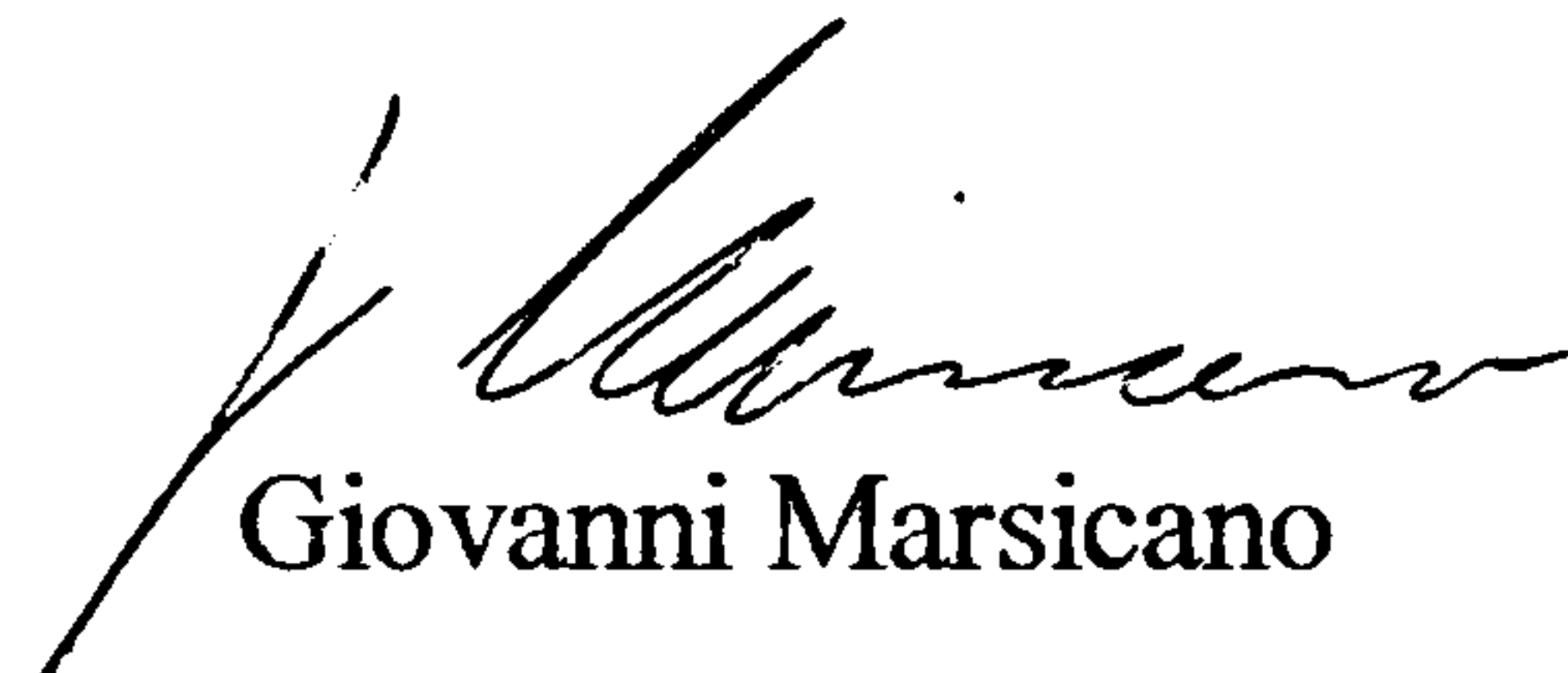
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List of abbreviations

2-AG	2-arachidonoylglycerol;
3V	third ventricle;
I-VI	layers of cerebral cortex;
AEA	anandamide;
AM 404	(allZ)-N-(4-Hydroxyphenyl)-5,8,11,14-eicosatetraenamide;
AO	anterior olfactory nucleus;
Aq	aqueductus;
AT	anandamide transporter;
ATG	transcription starting codon;
BLA	basolateral amygdaloid nucleus;
BMA	basomedial amygdaloid nucleus;
bp	base pairs;
BSA	bovine serum albumin;
C28	calbindin D28k;
CA1/CA3	CA1 and CA3 fields of hippocampus;
CaMK II α	calcium/calmodulin-dependent kinase II α
cAMP	cyclic adenosin-monophosphate;
CB1	cannabinoid receptor 1;
CBN	CB1-null mutant mouse line;
CCK	cholecystokinin;
cGMP	cyclic guanosinmonophosphate;
CNS	central nervous system;
CP 55,940	(-)- <i>cis</i> -3[2-Hydroxy-4-(1,1-dimethylheptyl)phenyl]- <i>trans</i> -4-(3-hydroxypropyl)cyclohexanol;
Cpu	caudate putamen;
Cre	<u>ca</u> using <u>re</u> combination;
CRT	calretinin;
Cx	cortex;
DAB	diaminobenzidin;
D2	dopamine receptor 2;
Δ^9 -THC	Δ^9 -tetrahydrocannabinol;
D-AP-5	D(-)-2-amino-5-phosphonopentanoic acid;
dATP	desoxyadenosintriphosphate;
dCTP	desoxycytosintriphosphate;
DG	dentate gyrus;
dGTP	desoxyguanosintriphosphate;
DMEM	Dulbecco's Modified Eagles Medium;
DMSO	dimethylsulfoxide;
DNA	desoxyribonucleic acid;
dpc	days post coitus;
DR	dorsal raphe;
dTTP	desoxythymintriphosphate;
EDTA	Ethylendiamintetracetate;
eNOS	endothelial nitric oxide synthase;
Ent	entorhinal/perirhinal cortex;
ESCs	embryonic stem cells;
FAAH	fatty acid amide hydrolase;
FCS	fetal calf serum;
floxed	<u>fl</u> anked by <u>loxP</u> sites;
Flp	flipase;

FRT	flipase recombination target;
G418	geneticin;
GABA	γ -aminobutyric acid;
GAD 65	glutamic acid decarboxylase 65k;
Glu	glutamate;
GP	globus pallidus;
Gr	granular layer of dentate gyrus;
HU 210	(6aR)- <i>trans</i> -3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol;
kb	kilobases;
IMD	medio/intermediodorsal thalamic nucleus;
i.p.	intraperitoneal;
iNOS	inducible nitric oxide synthase;
ISH	<i>in situ</i> hybridization;
KO	knock-out;
LA	left arm of homology;
LB	Luria Bertani medium;
Lhb	lateral habenula;
LIF	leukaemia inhibitory factor;
LMol	lacunosum-molecular layer of hippocampus;
L-NMMA	NG-monomethyl-L-arginine;
loxP	locus of crossover x in P1;
Ls	lateral septum;
M1/M2	primary and secondary motor cortex;
MAPK	mitogen activated protein kinase;
(+)MK-801	dizocilpine;
MO	medial orbital cortex;
Mol	molecular layer of hippocampus;
MPA	medial preoptic area;
Ms	medial septum;
Neo	neomycin phosphotransferase;
NMDA	N-methyl-D-aspartate;
nNOS	neuronal nitric oxide synthase;
NO	nitric oxide;
nor-BNI	nor-binaltorphimine;
OD600	optical density at 600 nm wave length;
Or	oriens layer of hippocampus;
ORF	open reading frame;
PAG	periaqueductal grey;
PBS	phosphate buffer saline;
PCR	Polymerase Chain Reaction;
PEG	polyethylenglicol;
pfus	plaque forming units;
PGK	phosphoglycerate kinase I;
Pir	piriform cortex;
pKS	pBlueScript KS (-);
Po	polymorph layer of dentate gyrus;
Prl	prelimbic cortex;
PV	parvalbumin;
PVT	paraventricular thalamic nucleus;
Py	pyramidal cell layer of hippocampus;
RA	right arm of homology;
Rad	stratum radiatum of hippocampus;

RNA	ribonucleic acid;
rpm	rotations per minute;
Rt	reticular thalamic nucleus;
S1FL/HL	somatosensory cortical areas, forelimb/hindlimb regions;
SDS	sodiumdodecylphosphate;
SEM	standard error of the mean;
SGr	subgranular layer of dentate gyrus;
SR 141716A	N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl;
TAE	Tris Acetate EDTA buffer;
Tm	approximate melting temperature for oligonucleotide;
Tu	olfactory tubercle;
VMH	ventromedial hypothalamic area;
w/v	weight per volume;
WT	wild-type;
ZI	zona incerta

Chapter 1

Introduction. Overview of the cannabinoid system

1.1 Historical aspects of *Cannabis sativa*: origin, diffusion and uses

Cannabis sativa (C.S.), also known as *Marihuana*, is one of the oldest cultivated plants and is considered one of the very first plants grown not specifically for their food content (reviewed by Peters & Nahas, 1999). C.S. was cultivated for two purposes: fibres for tissues and extracts for therapeutic and recreative purposes. First historical reports of the use of C.S. were found in China nearly 5000 years ago, where it was grown rather for fibers than for production of psychoactive extracts. From China, the use of C.S. moved to India, where it was mentioned as a sacred plant in the *Atharva Veda* (one of the holy books of hindu religion, 2000 BC). Herodotus reported the use of hemp among populations along the coasts of Caspian and Aral Seas (*Historiae III*, 440 BC). Romans and Greeks did not use C.S. as a pleasure-inducing drug as they preferred alcohol, but they cultivated the plant to obtain fibers to make ropes and sails. Some medical uses were described by the Greek physicians Dioscorides (*De materia medica*, ca. 60 AD) and Galen (129-210 AD). C.S. became very widespread in the Islamic world during the Middle Age. Through the Arab invasions (ninth to twelfth century AD) its use was diffused to all the southern Mediterranean coast, from Egypt to Morocco (Nahas, 1982). Arab physicians such as al-Razi (900 AD) and al-Badri (1251 AD) described many details of medical uses of C.S. and recommended it for the ear, for dissolving flatulence, to cure epilepsy and to induce appetite (references from Peters & Nahas, 1999).

In the first half of the 18th century, the British physician O'Shaughnessy introduced C.S. into British medicine, after observing its use in India. He and other British physicians prescribed C.S. extracts for the treatment of rabies, rheumatism,

epilepsy, tetanus and as antialgesic (Nahas, 1973). In 1840, Jacques-Joseph Moreau, considered as the father of modern psychopharmacology, observed experimentally the properties of C.S. (Moreau, 1845). He compared the effects of C.S. extracts on himself to the symptoms observed with his mental patients. He described eight symptoms present either after C.S. intoxication or in mental illness: feeling of bliss, dissociation of ideas, errors of time and space appreciation, exacerbation of the sense of hearing, fixed ideas, disturbances of emotions, irresistible impulses and illusions. Interestingly, from such observations, he became convinced of the organic nature of mental illness and that "hashish [is] a unique powerful tool to deeply explore the mental pathologies".

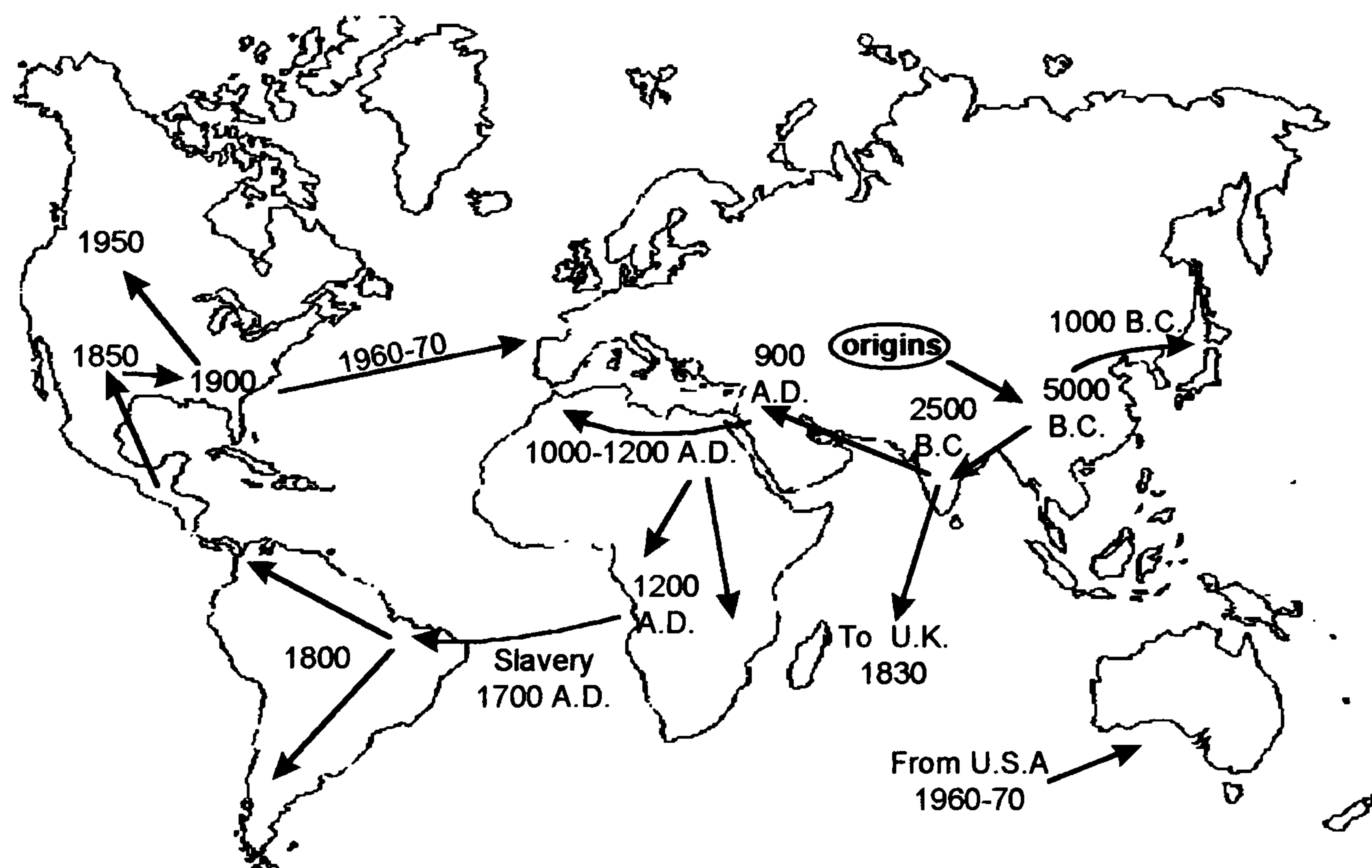


Fig. 1.1. Schematic representation of the propagation of *Cannabis sativa*, through the ages.

Marihuana smoking for recreational purposes reached the Americas via the African slaves deported to Brazil in the 17th century by the Portuguese. From Brazil, the use of "grass" (the translation of the Arabic word *hashish*) spread to central America and from there reached the U.S.A., where it was first the typical drug of black jazz musicians in the early 20th century. In the 1960s, it became widely diffused among university students and was then returned eastwards to Europe (Fig. 1.1). Today, C.S. extracts are the most widely used illicit drugs in western world. However, the therapeutic potential,

after many decades of oblivion, are again the focus of attention of scientific as well as social and political communities.

1.2 Hallmarks in cannabinoid research

Despite the long history, the mechanisms of action of C.S. derived drugs have not yet been completely understood. However, in the last decades, an impressive body of studies has led to many and important clarifications. An indicative progression of the scientific interest in C.S. can be obtained by a simple counting of the publications during the last three decades (Fig. 1.2).

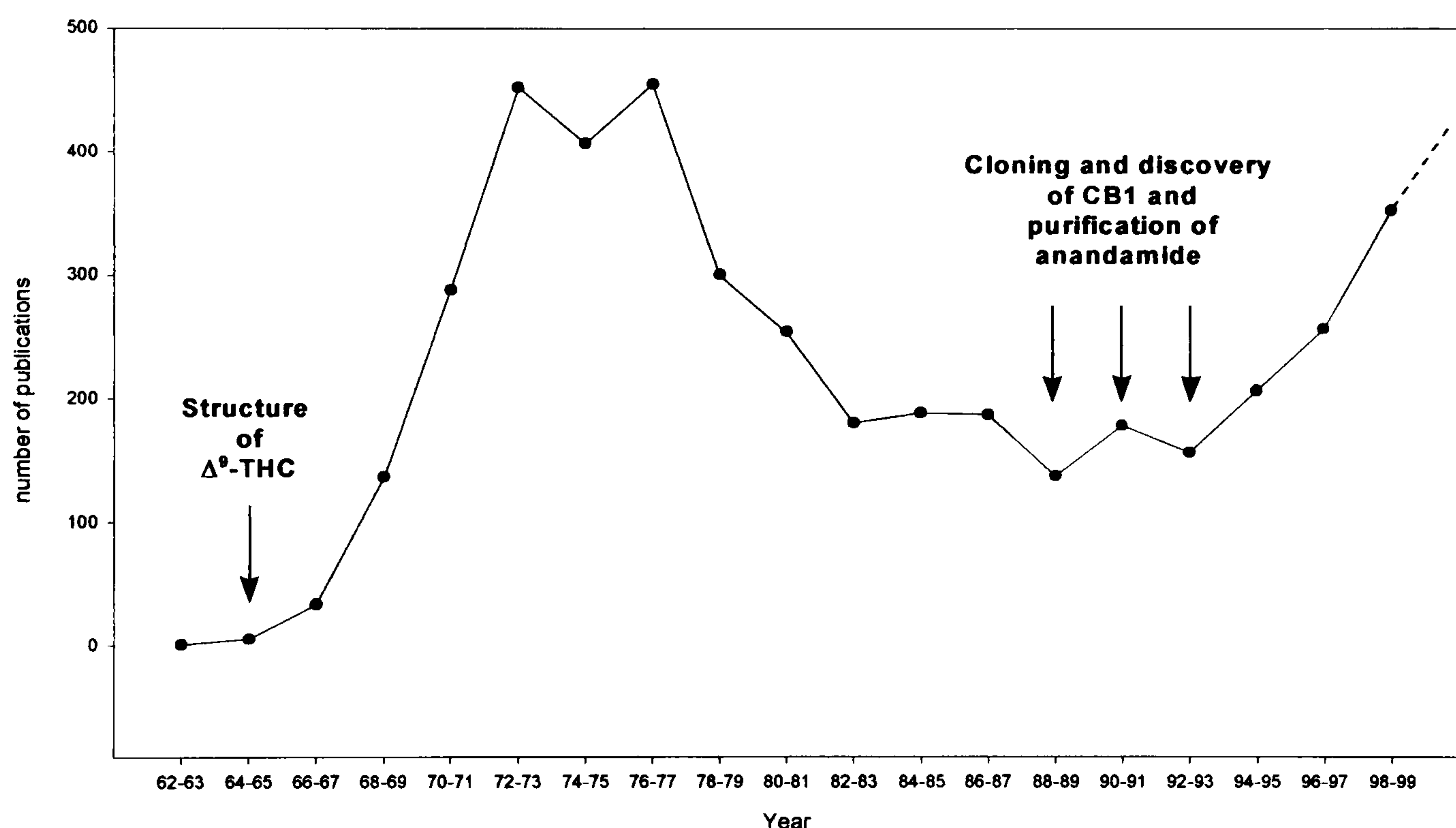


Fig 1.2. Number of publications produced in recent decades, in relation to cannabinoid research. The values were obtained with a Medline search (<http://www.ncbi.nlm.nih.gov/entrez/query>), using as keywords "cannabinoid OR marihuana" and limiting the search to two years' periods. Key discoveries are indicated.

One of the first and most important steps in recent *marihuana* research was the purification of the major psychoactive compound in C.S., (-)- Δ^9 -tetrahydrocannabinol (Δ^9 -THC; Mechoulam & Gaoni, 1965; Petrzilka & Sikemeier, 1967). Since those pioneering studies, the number of published papers increased exponentially, indicating a high interest in the chemistry, biochemistry, biology and pharmacology of C.S.

derivatives (Fig 1.2). During the late 70s and the early 80s the number of publications seemed to decrease slightly, likely reflecting major difficulties in defining precise mechanisms of action of Δ^9 -THC and natural and synthetic cannabinoids. Due to the high lipophilicity of cannabinoid compounds, two mechanisms were postulated: a direct interaction with the cell membranes (Hillard et al., 1985) or an interaction mediated through still undiscovered membrane receptors (Howlett et al., 1986). Indicative data were present for both hypotheses. However, in 1988, the availability of new potent synthetic cannabinoid compounds, such as CP-55,940 (Johnson & Melvin, 1986), led to the discovery of a typical receptor-mediated interaction between cannabinoid compounds and biological tissues (Howlett et al., 1988). Such a finding, even though far from explaining all the effects of cannabinoids, represented a new impulse to the research. Two years later, Matsuda et al. (1990) cloned the first cannabinoid receptor (CB1) and opened the way to the molecular approach to the biology of cannabinoids. CB1 was described as a G protein coupled receptor and was found to be expressed mainly in the brain, indicating a function in central effects of cannabinoids. Later, Munro et al. (1993) cloned a second receptor, named CB2, also able to bind cannabimimetic compounds, whose expression is limited to cells and tissues of the immune system. In this way, the early 90s paved the way towards the understanding of the mechanism(s) of action of exogenously administered C.S. derivatives and synthetic cannabinoids. However, one important piece of the puzzle was still missing: the endogenous ligands of the receptors, the so-called "endocannabinoids". One did not have a long time to wait to get such an answer. Devane et al. (1992) purified from porcine brains a novel compound that was able to bind and stimulate cannabinoid receptors. This compound is an amide of arachidonic acid and was named "anandamide", from the Sanskrit word for "bliss", *ananda*. Later, another endogenous ligand was described, 2-arachidonyl glycerol (Mechoulam et al., 1995; Sugiura et al., 1995). Furthermore, an active system for

anandamide uptake was uncovered in cells (Hillard et al., 1997; Beltramo et al., 1997; Bisogno et al., 1997a), and specific pathways were found that produce and degrade the endocannabinoids (Di Marzo et al., 1994). Such an impressive efflux of new data and discoveries led the "cannabinoid researchers" to postulate the existence of a new signalling system, made by endogenous compounds, their specific receptors and their specific mechanisms for production, release, uptake and degradation (reviewed by Di Marzo et al., 1998). Moreover, the functional interactions of the "cannabinoid system" with other physiological systems appear to be more and more relevant, participating in the maintenance of the physiological status and possibly being dysregulated in pathophysiological conditions. As mentioned before, CB1 and CB2 are expressed mainly in the brain and in the immune system, respectively. Therefore, it appears that two distinct (although likely interacting) "cannabinoid systems" exist, one related to brain function and the other to immunological activities.

1.3 CB1, the "brain type" cannabinoid receptor

1.3.1 Mechanisms of action

Despite the possibility that cannabinoids could exert some of their actions through non receptor-mediated mechanisms, there is general agreement that most of such actions are mediated by specific receptors. At the moment, two "cannabinoid receptors" have been cloned: the "brain type" CB1, expressed in the central nervous system, but also in many peripheral organs (Matsuda et al., 1990; Shire et al., 1995) although at lower levels and CB2, whose expression is limited to cells and organs of the immune system (Munro et al., 1993). Both CB1 and CB2 are seven transmembrane G protein coupled receptors, generally coupled to $G_{i/o}$ proteins. CB2 was cloned from a leukaemic cell line and has a relatively low sequence identity with CB1 (44% overall the whole protein, 68% in the transmembrane regions) (Munro et al., 1993). CB1 has been extensively characterised. *In vitro* studies, using primary neuronal cultures, neuronal cell

lines that endogenously express CB1, heterologous expression of the receptor in other cell lines, or brain slices, have revealed that the intracellular signal transduction pathways stimulated by CB1 activation are dependent on the system used. However, CB1 is believed to exert its functions presumably through two main intracellular pathways: inhibition of adenylate cyclase and alterations of ion channels activity. However, in the recent years, also other intracellular pathways have been shown to be triggered by CB1.

1.3.1.1 Inhibition of adenylate cyclase

A reversible, dose-dependent and stereoselective inhibitory action of Δ^9 -THC on adenylate cyclase activity in neuroblastoma cells was one of the key observations that led to the discovery of the existence of cannabinoid receptors (Howlett & Fleming, 1984; Howlett et al., 1986; Bidaut-Russell et al., 1990). Such an inhibition is mediated by inhibitory G proteins ($G_{i/o}$), because this effect can be blocked by *pertussis* toxin in neuroblastoma cells, in membranes derived from mammalian brain and in primary neuronal cultures (Howlett et al., 1986; Bidaut-Russell et al., 1990; Bouaboula et al., 1995). Pertussis toxin is able to prevent the dissociation of the α and β/γ subunits of $G_{i/o}$, thereby blocking the G protein-mediated inhibition of adenylate cyclase. Activation of G proteins was also shown by receptor-stimulated [35 S]GTP γ S binding to brain-derived membranes and to brain sections (Sim et al., 1995; Breivogel et al., 1997). Interestingly, the regional distribution of CB1 receptor as revealed by radioligand binding, and the activation of G proteins by cannabinoid agonists are very similar, indicating that presumably all CB1 receptors are able to activate G proteins. However, quantitative differences suggest that the receptor can have different coupling efficiencies in various brain regions (Sim et al., 1995; Childers & Breivogel, 1998; Ameri, 1999). The adenylate cyclase-cAMP-protein kinase A (PKA) pathway is one of the key mechanisms in neuronal physiology. As an example, cAMP-dependent gene expression appears to be universally required for establishing long-term memory (Silva et al., 1998; Alberini,

1999). Therefore, the inhibition of this pathway by cannabinoids acting at CB1 can explain many of the pharmacological effects of cannabinoids, such as the inhibition of memory formation (Hampson & Deadwyler, 1998). The coupling of CB1 to $G_{i/o}$ proteins is considered one of the main mechanisms of action of the receptor, but evidence also exists indicating that different G protein subtypes are involved in CB1 signal transduction. As an example, coupling with G_s proteins was described in primary striatal neurons, after a simultaneous stimulation of CB1 and D2 dopamine receptors (Glass & Felder, 1997). Another important element of the CB1 signal transduction pathways is the ability of the receptor to sequester G proteins, rendering them unavailable for the action of other G protein coupled receptors (Vasquez & Lewis, 1999).

1.3.1.2 Modulation of ion channels

$G_{i/o}$ is able to couple seven transmembrane receptors not only to adenylate cyclase, but also to ion channels. Studies on neuroblastoma cell lines and on primary neurons expressing CB1 revealed that cannabinoid receptor activation inhibits voltage-activated inward calcium currents (Caulfield & Brown, 1992; Mackie & Hille, 1992). This effect appears to be mediated by $G_{i/o}$, because it is blocked by *pertussis* toxin. In experiments using inhibitors of different calcium channel subtypes, it was shown that N-type and P/Q-type calcium channels are mainly involved in this effect of CB1 (Mackie & Hille, 1992). Moreover, the addition of non-hydrolysible analogues of cAMP or inhibitors of phosphodiesterase did not alter the inhibitory effect of CB1 onto calcium channels, thus indicating an adenylate cyclase-independent mechanism. CB1 was shown to regulate also the actions of potassium channels. Early studies revealed that cannabinoids acting at CB1 can modulate two potassium channels, the inwardly rectifying potassium (K_{ir}) channel and the voltage-dependent A-type potassium channel. Inwardly rectifying potassium currents are enhanced by cannabinoids in a dose- and *pertussis* toxin-dependent manner, thus indicating a $G_{i/o}$ -dependent process (Henry &

Chavkin, 1995; Mackie et al., 1995). Interestingly, a recent report by Garcia et al. (1998) showed that cannabinoid actions on K_{ir} and on P/Q-type calcium channels can be strongly attenuated by phosphorylation of CB1 at a single serine residue (S317) in the third cytoplasmic loop of the receptor by the action of protein kinase C (PKC), thus constituting a putative regulatory system of CB1. Deadwyler et al. (1993) have shown that the voltage-dependent potassium A currents (K_A) are significantly enhanced in a concentration-dependent manner by CB1 agonists in primary hippocampal cells.

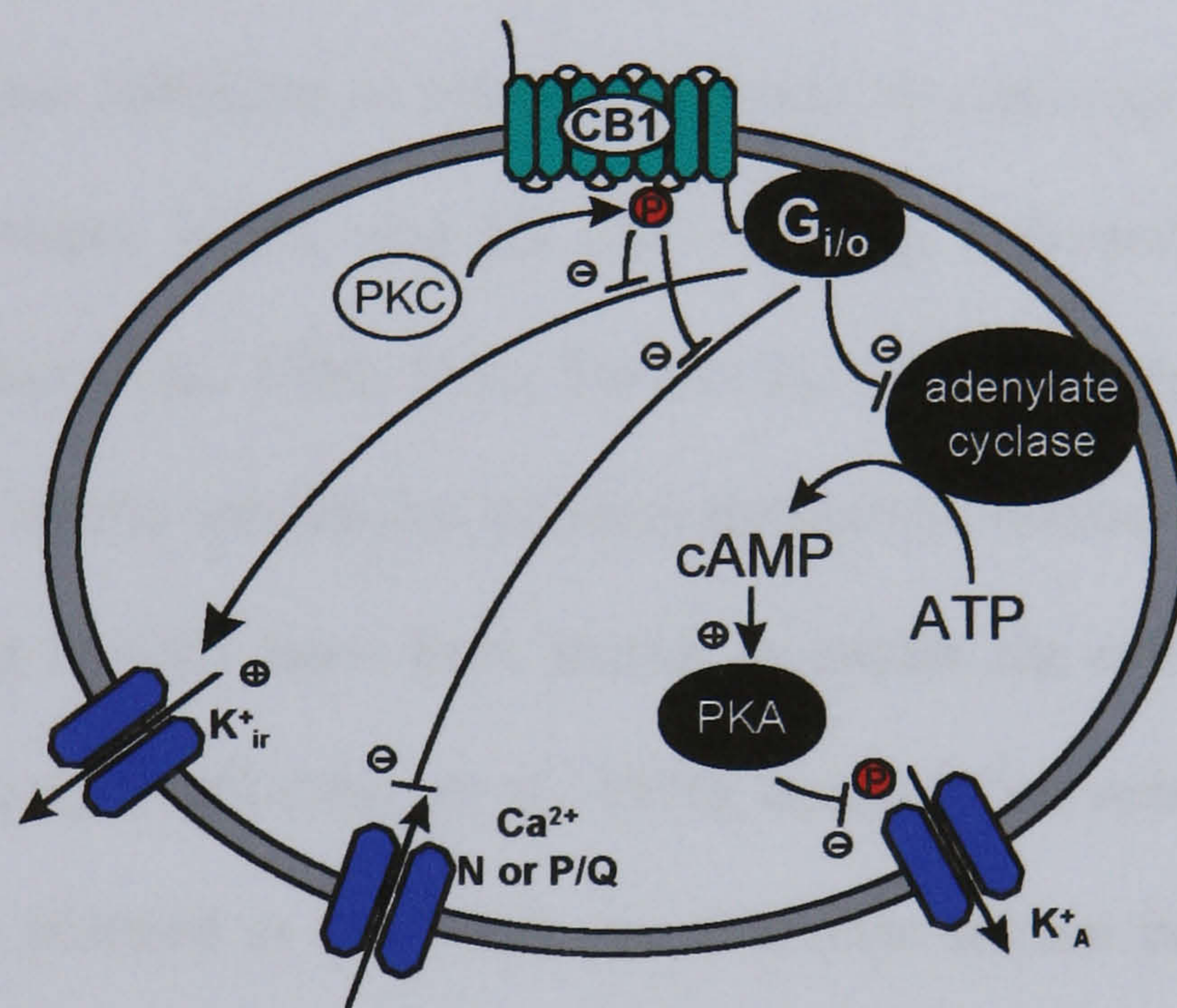


Fig. 1.3. Schematic representation of the main effects of CB1 on ion channels. Activation of CB1 leads to the stimulation of $G_{i/o}$ proteins that, in turn, inhibits the adenylate cyclase-mediated conversion of ATP into cAMP. cAMP molecules can bind the regulatory subunits of protein kinase A (PKA) and cause the liberation of the catalytic subunits. Active PKA can phosphorylate A-type potassium channels (K_A^+), causing a decrease of the current. Given the negative effect of CB1 on adenylate cyclase, the final result is an activation of A-type potassium channels. $G_{i/o}$ activated by CB1 can also directly inhibit N- or P/Q-type calcium channels and activate inwardly rectifying potassium channels (K_{ir}). These last two effects are controlled by protein kinase C (PKC), that, when activated, can phosphorylate CB1 in the third cytoplasmic loop and uncouple the receptor from the ion channels.

The activation of A-type potassium channels (K_A) by CB1 is dependent on the inhibition of cAMP synthesis with the subsequent inhibition of a protein kinase A (PKA)-mediated phosphorylation of the channel, but not on a direct coupling of $G_{i/o}$ proteins to

the A-type potassium channel (Deadwyler et al., 1993; Hampson et al., 1995; Childers and Deadwyler, 1996).

Thus, cannabinoids acting at CB1 are able to decrease the Ca^{2+} influx by inhibiting N- and P/Q-type calcium channels and to increase the efflux of K^{+} ions by activating inwardly rectifying potassium currents and A-type potassium channels (Fig. 3). Considering that i) the calcium channels are mainly located presynaptically and are necessary for evoked neurotransmitter release in the CNS (Takahashi & Momiyama, 1993; Wheeler et al., 1994; Ameri, 1999), ii) the activation of potassium channels can amplify a presynaptic inhibition of calcium channels by reducing the duration of the action potential (Ameri, 1999), and iii) CB1 is highly expressed in the presynaptic terminals (Herkenham et al., 1990, 1991; Tsou et al., 1998a), it appears likely that CB1 plays a major role in the modulation of neurotransmitter release at synapses. Indeed, cannabinoids acting at CB1 have been shown to inhibit the release of glutamate in cultured rat hippocampal cells (Shen et al., 1996), acetylcholine release in superfused rat hippocampal slices (Gifford et al., 1997), noradrenaline release in slices from human, guinea pig and rat hippocampus (Schlicker et al., 1997), and GABA release from rat hippocampal slices (Katona et al., 1999). Thus, the presynaptic inhibition of several neurotransmitters by cannabinoids can be considered as a plausible cellular mechanism underlying the psychoactive actions of cannabinoids.

1.3.1.3 Other pathways triggered by CB1

Inhibition of adenylate cyclase, stimulation of A-type and inwardly rectifying potassium currents, and inhibition of N- and P/Q-type calcium channels are considered as the main intracellular events induced by CB1 in neurons. However, recent observations revealed additional regulatory mechanisms. In electrophysiological experiments on rat hippocampal slices, Schweitzer (2000) showed that cannabinoids acting at CB1 are able to decrease the activity of postsynaptic potassium-persistent voltage-dependent M-

currents in CA1 hippocampal pyramidal cells. This would indicate that cannabinoids may specifically increase the excitability of postsynaptic cells. Moreover, using microdialysis techniques, very recent results from Acquas et al. (2000) indicate an increase of acetylcholine release in hippocampus and frontal cortex of freely moving rats after *in vivo* treatment with low doses of cannabinoid agonists. It is therefore possible that cannabinoids acting at CB1 can exert different intracellular effects in different brain areas or at different concentrations. Netzeband et al. (1999) showed that in primary cerebellar granule cells, CB1 is able to enhance the peak amplitude of the Ca^{2+} response elicited by stimulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (NMDARs). This effect appears to involve pertussis toxin-sensitive G proteins ($\text{G}_{i/o}$) and the phospholipase C (PLC)/inositol 1,4,5-trisphosphate (IP3) pathway, and finally results in an increased Ca^{2+} release from intracellular stores. These data are very interesting in light of the facts that i) PLC and IP3 can trigger the activation of PKC (Schwartz & Kandel, 1991) and ii) PKC can inhibit, by phosphorylation of the third intracellular loop, the effects of CB1 on calcium channels and inwardly rectifying potassium channels (Garcia et al., 1998). Therefore, it can be speculated that, in certain conditions (i.e. upon NMDAR activation by glutamate), cannabinoids acting at CB1 might "switch" their effects on calcium mobility, by enhancing the release of Ca^{2+} from intracellular stores, and by inhibiting, following PKC phosphorylation, their own effects on calcium channels and inwardly rectifying potassium channels. The involvement of IP3 in the signal transduction cascade of CB1 was also very recently shown by Gomez Del Pulgar et al. (2000). Using CB1-transfected Chinese hamster ovary cells (CHO) and human glioma cells, these authors showed that cannabinoids can stimulate protein kinase B/Akt (PKB) in a CB1-, $\text{G}_{i/o}$ - and IP3-dependent manner, thus, indicating a novel potential mechanism of cannabinoid action. Another potentially very interesting intracellular cascade triggered by CB1 is the mitogen activated protein kinase (MAPK)

pathway. Bouaboula et al. (1995) showed that cannabinoid treatment of CB1 transfected CHO cells was able to stimulate the phosphorylation of MAPKs in a receptor-dependent, *pertussis* toxin-sensitive and cAMP-independent manner. MAPKs pathway is very important in many aspects of neurophysiology, from differentiation and survival of neurons (Fukunaga & Miyamoto, 1998), to the induction of important forms of neuronal plasticity, such as long-term memory (Orban et al., 1999). However, thus far, the activation of MAPK by CB1 receptor has only been found in non-neuronal cell lines artificially transfected with CB1. Therefore, it is still uncertain whether it plays role in the CNS under physiological conditions.

In conclusion, the inhibition of adenylate cyclase, the activation of A-type and inwardly rectifying potassium channels and the inhibition of N- and P/Q-type calcium channels appear as the prominent mechanisms through which cannabinoids acting at CB1 exert their intracellular effects. Nevertheless, other pathways can be, in different conditions, activated by CB1, and the direction of the research in the near future will likely focus not only on finding novel mechanisms of action, but also on defining the cellular status leading to the activation by cannabinoids of either of the intracellular pathways. Already the CB1 signal transduction changes caused by simultaneous dopamine D2 receptors (Glass & Felder, 1997) and NMDAR activation (Netzeband et al., 1999) represent very interesting functional receptor cross talks that might contribute to the understanding of the different, and sometimes apparently divergent, effects of cannabinoids.

1.3.2 Distribution of CB1 in the mammalian brain

After the discovery of the existence of a cannabinoid receptor in the brain, the cannabinoid receptor distribution was first shown by autoradiography of ligand-receptor binding on slide-mounted rat brain sections, with the radiolabeled agonist [³H]CP 55,940 (Herkenham et al., 1990, 1991). Autoradiographic experiments using another radioligand

in rats, [³H]WIN 55,212-2, and [³H]CP 55,940 in humans, confirmed the general distribution of the receptor (Jansen et al., 1992; Glass et al., 1997a). It became quickly apparent that the quantity of receptor in the brain is very high, thus, CB1 can be considered one of the most abundant G-protein coupled receptors in mammalian brain (Herkenham et al., 1990), comparable in quantity and density with glutamate receptors. After the cloning of CB1 (Matsuda et al., 1990), the distribution of CB1 was investigated at the mRNA level, by *in situ* hybridization (ISH) in rodents (e.g. Matsuda et al., 1993; Marsicano & Lutz, 1999) and in humans (Mailleux et al., 1992; Westlake et al., 1994). More recently, anti-CB1 antibodies became available, thus the immunohistochemical (IHC) distribution of the receptor was determined (Tsou et al., 1998a; Pettit et al., 1998). Agonist-stimulated [³⁵S]GTP γ S binding to slide-mounted sections (Sim et al., 1995) is another interesting method that has been used to define the functional neuroanatomy of the brain type cannabinoid receptor (Breivogel & Childers, 1998).

The highest density of cannabinoid receptors has been demonstrated in basal ganglia (substantia nigra, globus pallidus, entopeduncular nucleus and dorsolateral caudate putamen) and in the cerebellum. In these areas, discrepancies between mRNA (ISH) and protein (IHC and ligand binding) expression were observed, thus indicating the presence of the receptor on distal neuronal projections. As an example, substantia nigra pars reticulata does not contain CB1 mRNA, but is one of the most densely stained regions in immunohistochemical and ligand binding experiments (Mailleux & Vanderhaeghen, 1992; Westlake et al., 1994; Matsuda et al., 1993; Tsou et al., 1998a).

High densities of binding were also described in the CA pyramidal cell layers of hippocampus (Herkenham et al. 1990). Such binding was shown by immunohistochemistry to be due to a dense plexus of immunoreactive fibres surrounding the cell bodies of pyramidal cells which appear *per se* devoid of CB1 protein (Tsou et al.,

1998a). However, pyramidal cells of hippocampus have been shown to express low but significant levels of CB1 mRNA (Matsuda et al., 1993; Marsicano & Lutz, 1999; Chapter 2), thus again indicating the possibility that CB1 protein is localised on distal projections of pyramidal CA hippocampal neurons. In hippocampus, neocortex (layers II, III, V and VI), entorhinal cortex, amygdaloid region (basomedial and basolateral amygdala), anterior olfactory nucleus, olfactory tubercle and piriform cortex, CB1 is expressed (at mRNA and protein levels) both in scattered cells containing very high levels of the receptor and in more diffuse low-expressing neurons (Matsuda et al., 1993; Tsou et al., 1998a). In these cortical regions, scattered highly expressing cells are likely to be GABAergic interneurons, while diffuse low-expressing cells are probably principal glutamatergic neurons (Katona et al., 1999; Marsicano & Lutz, 1999; Chapter 2). Other forebrain areas that contain low levels of CB1, generally uniformly distributed, are the ventromedial hypothalamic area and some thalamic nuclei. In the hindbrain, beside the molecular and granular layers of cerebellum expressing high levels of the receptor, CB1 is present at low levels in some nuclei of brain stem, such as the periaqueductal gray (Matsuda et al., 1993; Tsou et al., 1998a).

Functional mapping by agonist-stimulated [³⁵S]GTP γ S binding using different CB1 agonists, revealed that cannabinoid activation of G-proteins occurs with the same regional distribution as the receptors (Breivogel et al., 1997; Breivogel & Childers, 1998). However, in some regions, the ratio between estimated amount of receptor and the G protein activation is not always constant, thus indicating regional differences in receptor coupling efficiencies (Breivogel & Childers, 1998). This is important to be considered, because sometimes cannabinoids seems to exert pharmacological effects that involve regions where the density of CB1 is very low (e.g. pain modulation mediated in the periaqueductal gray). Therefore, the activity of cannabinoids at CB1 cannot be predicted solely based on relative receptor density, but other factors, such as receptor

coupling efficiency or different functional conditions of the receptor (e.g. phosphorylation, see above, 1.3.1 "Mechanisms of action") must be taken in account.

1.3.3 CB1 ligands

1.3.3.1 *The problem of nomenclature.*

C. sativa contains more than 60 compounds belonging to the chemical family of "cannabinoids" (Iversen, 2000). Among them, Δ^9 -THC was described as the most psychoactive one and was shown to bind and activate CB1 (Mechoulam and Gaoni, 1965; Howlett et al., 1986; Matsuda et al., 1990). Considering this aspect, some problems arise for the nomenclature of cannabinoid compounds. Classically (and more correctly), the term "cannabinoid" should be referred to a class of compounds sharing some structural characteristics as three ring structure, the lack of nitrogen atoms and the presence of a pentyl side chain regardless whether derived from *C.S.* (Nahas et al., 1999). However, with the discovery of the cannabinoid receptors and the development of new synthetic agonists and antagonists, the term "cannabinoid" has inevitably extended its meaning. In its general use, it names all the compounds derived from *Cannabis sativa* possessing the above mentioned structural characteristics and all the molecules (agonists or antagonists, natural or synthetic) that are able to bind to CB1 and/or CB2 receptors. The discovery of endogenous ligands for CB1 and CB2 with the related mechanisms for synthesis, release, uptake and degradation (see below), have further increased the complexity of the "cannabinoid" nomenclature. Again, commonly, "endocannabinoids" are called the endogenous ligands of CB1 and CB2 (Di Marzo et al., 1998), and, in a general sense, "cannabimimetic" compounds are all the chemicals (natural or synthetic) that are able to interfere with the metabolism of endocannabinoids, therefore increasing or decreasing the activity of these compounds. This classification is, of course, conventional and limited for many aspects, but it is the most common nomenclature used in the recent literature. Therefore, this will be the adopted convention, in this thesis.

1.3.3.2 CB1 agonists

From the chemical point of view, CB1 agonists can be classified into four groups (Pertwee, 1997; Fig 1.4): (i) "classical cannabinoids", that are dibenzopyran derivatives and include Δ^9 -THC. (ii) "nonclassical cannabinoids", developed by Pfizer (USA), which contain bicyclic and tricyclic analogues of Δ^9 -THC that lack a pyran ring (Johnson and Melvin, 1986). The most important compound of this family is CP 55,940, used for the demonstration of the existence of the cannabinoid receptors (Howlett et al., 1986). (iii) Aminoalkylindoles, developed by Sterling Winthrop (USA) (Pacheco et al., 1991). The members of this group are structurally quite different from "classical" and "nonclassical" cannabinoids. The prototype of this group is WIN 55,212-2. (iv) "Eicosanoids", that are derivatives of arachidonic acid and were discovered as endogenous ligands of the cannabinoid receptors (Devane et al., 1992). Prototypes of this group are anandamide (arachidonylethanolamide, AEA) and 2-arachidonylglycerol, the two major endocannabinoids so far discovered in mammals.

1.3.3.3 CB1 antagonists

As soon as cannabinoid receptors were discovered, several newly synthesised compounds were tested as putative specific antagonists of CB1 or CB2. The first compounds to be shown to have some antagonistic properties at cannabinoid receptors were members of the aminoalkylindoles group, WIN 56,098 (Pacheco et al., 1991) and 6-bromopravadoline (WIN 54,461; Casiano et al., 1991). However, the initial compound is rather weak antagonist (K_d in the order of micromolar), while the latter compound, even though more potent, has been shown to act, in some cannabinoid tests, as a partial agonist (Pertwee, 1997). Another aminoalkylindole compound, AM630 (6-iodopravadoline; Pertwee et al., 1995) has good potency, but again can act as partial agonist in some preparations and is not effective *in vivo* (Eissenstat et al., 1995).

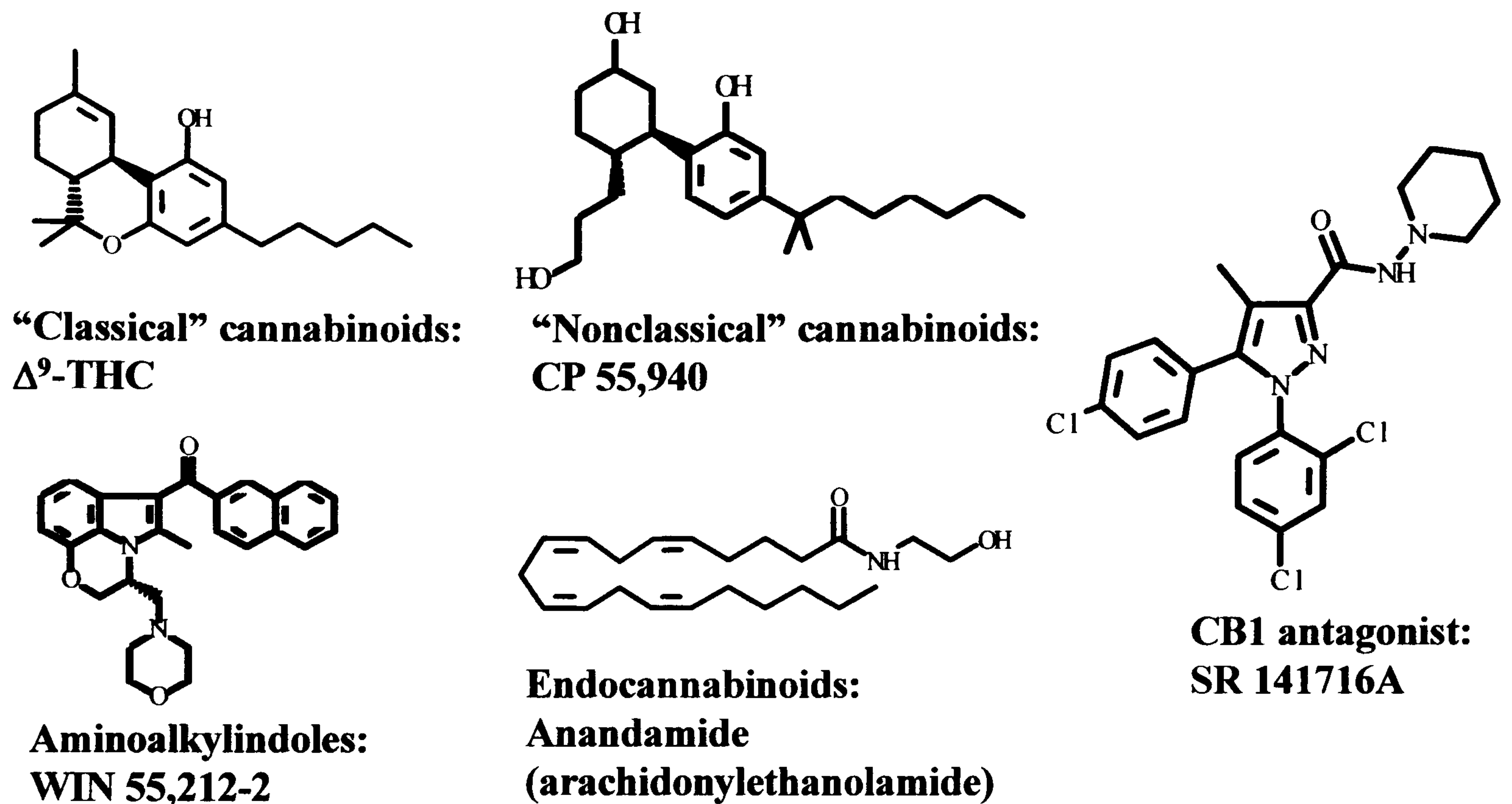


Fig 1.4. Structures of CB1 ligands.

By far, the most potent and well-characterised CB1 antagonist is SR141716A (Rinaldi-Carmona et al., 1994; Fig 1.4). This compound is able to displace [3 H]CP 55,940 from specific binding sites (K_d = ca. 2 nM) and is a potent antagonist of several of the typical effects of cannabinoids, both *in vitro* and *in vivo*. Another interesting property of SR141716A is the high specificity for CB1, having little or no affinity for CB2 and for a wide range of other membrane receptors (Rinaldi-Carmona et al. 1994; Compton et al., 1996). More recently, a CB2 specific antagonist was developed by the same group, SR 144528 (Rinaldi-Carmona, 1998). Both these antagonists were shown to act, in some conditions, in an opposite way as cannabinoid receptor agonists (inverse agonism) (Rinaldi-Carmona, 1998; Portier et al., 1999). SR141716A, in particular, is able to increase locomotor activity in mice (Compton et al., 1996), improve some forms of short-term memory in rats and mice (Terranova et al., 1996), and enhance the forskolin-induced production of cAMP in cells transfected with CB1 (Felder et al., 1995). Two different mechanisms could explain these observations. First, cannabinoid receptors might be tonically stimulated by endogenous ligands, whose action is blocked

by the addition of the antagonist. Second, the receptor might be present in two different conformational state, one precoupled to and the other uncoupled from the effector system(s). In this case, the SR compounds could produce their effects in absence of other drugs, because they bind preferentially to the receptor in the uncoupled state, thus shifting the equilibrium away from the precoupled status (Pertwee, 1997; Shire et al., 1999). This second mechanism is very interesting, also in view of the regional differences in receptor coupling efficiencies of CB1 (Breivogel & Childers, 1998), once more pointing to the importance of the functional status of the receptor in different anatomical regions and in different cellular conditions.

1.4 The cannabinoid system

With the discovery of cannabinoid receptors (Matsuda et al., 1990; Munro, 1993), it became clear that natural and synthetic cannabinoids were likely to interfere with an endogenous neuronal system, in analogy, for example, to the opiates with the endogenous opioid system. In 1992, the first endogenous ligand of CB1 was purified from porcine brain (Devane et al., 1992). The compound is an amide of arachidonic acid with ethanolamine and was named anandamide from the Sanskrit word "ananda" meaning "bliss". Early studies showed that anandamide (also called arachidonylethanolamide, AEA) is able to induce behavioural effects typical for Δ^9 -THC and other CB1 agonists in rodents, i.e. inhibition of locomotor activity, catalepsy, analgesia on a hot plate and rectal hypothermia (Fride & Mechoulam, 1993). After the discovery of anandamide, other metabolites were characterised in the brain with similar agonistic effects at cannabinoid receptors. These are all polyunsaturated fatty acid derivatives with no higher efficacy than anandamide and comprise docosatetraenylethanolamide, di-homo- γ -lineoylethanolamide (Hanus et al., 1993), and 2-arachidonylglycerol (2-AG, Mechoulam et al., 1995; Sugiura et al., 1995).

1.4.1 Synthesis of endocannabinoids

Studies carried out in rat neurons demonstrated that synthesis and release of anandamide, whose basal levels in the brain are low as compared to most neurotransmitters, could be stimulated by treatment with depolarising agents such as ionomycin, 4-aminopyridine, kainate and high extracellular K^+ concentrations (Di Marzo et al., 1994).

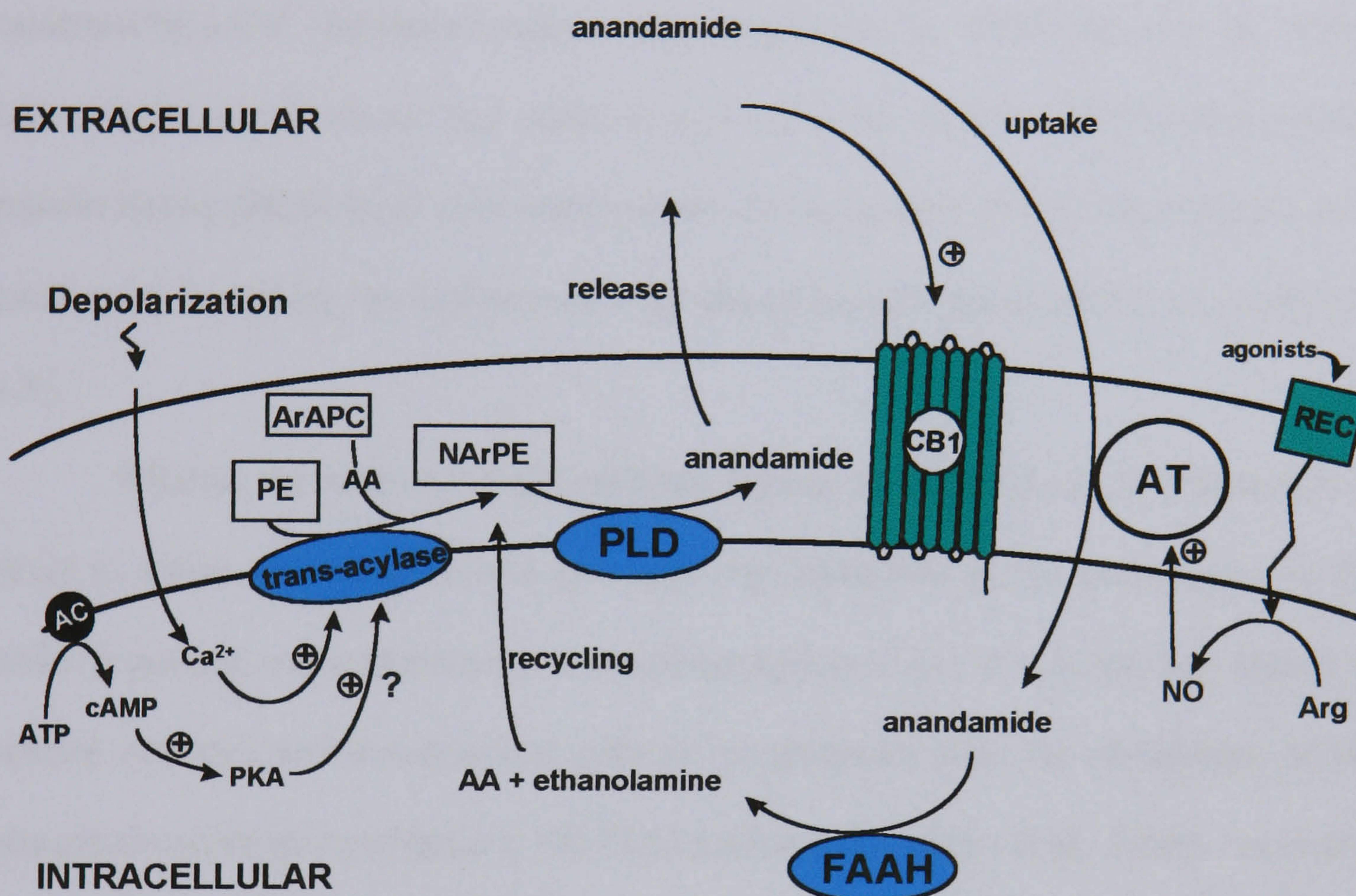


Fig. 1.5. Schematic representation of the main mechanisms involved in the synthesis, release, re-uptake and degradation of anandamide. AA, arachidonic acid; AC, adenylyl cyclase; ArAPC, *sn*-1,2-di-arachidonoyl-phosphatidylcholine; AT, "anandamide transporter"; FAAH, fatty acid amide hydrolase; NArPE, N-arachidonoyl-phosphatidylcholine; NO, nitric oxide; PE, phosphatidyl-ethanolamine; PKA, cAMP-dependent protein kinase; PLD, N-acyl-phosphatidyl-ethanolamine-selective phospholipase D.

Neurons and astrocytes are also able to rapidly uptake and degrade anandamide, forming arachidonic acid and ethanolamine (Di Marzo et al., 1994; Deutsch & Chin, 1993). In this view, anandamide appears to act as a classical neurotransmitter, with depolarisation-induced release, action at specific receptors, (re)-uptake by cells and intracellular degradation. However, differently from classical neurotransmitters,

anandamide and other endocannabinoids are not stored in presynaptic vesicles, but are thought to be synthesised "on demand" from membranous precursors. Anandamide, in particular, is likely to originate from the phospholipase D (PLD)-catalysed hydrolysis of a phospholipid precursor, N-arachidonyl-phosphatidyl-ethanolamine (NArPE; Di Marzo et al., 1994; 1998). NArPE is produced by the transfer of an arachidonate moiety from 1,2-*sn*-di-arachidonyl-phosphatidylcholine (ArAPC) to phosphatidyl-ethanolamine (PE), catalysed by a Ca^{2+} -dependent trans-acylase (Sugiura et al., 1996; Cadas et al., 1996). Interestingly, it was shown that stimulation of adenylate cyclase and cAMP-dependent protein kinase (PKA) leads to an enhancement of the activity of the trans-acylase, thus, presumably increasing the final amount of produced anandamide (Cadas et al., 1996) (Fig 1.5).

Whereas the main metabolic pathway leading to the synthesis of anandamide is likely as above described, several pathways may contribute to the biosynthesis of the other important endocannabinoid, 2-arachidonylglycerol (2-AG). 2-AG was shown in several neuronal and non-neuronal cells to be produced after the stimulation of the phosphoinositide-phospholipase C (PI-PLC) pathway (Di Marzo et al., 1996). According to this pathway, 2-AG would be formed by the sequential hydrolysis of phosphatidyl inositols and diacylglycerols (DAGs) catalyzed by the G-protein-coupled PI-PLC and *sn*-1-selective DAG lipase, respectively (Di Marzo, 1999). However, also other pathways were proposed, where 2-AG is the result of a depolarisation-induced process. After stimulation with ionomycin, DAGs serving as 2-AG precursor can be produced by cortical neurons through the PI-PLC pathway (Stella et al., 1997) and by mouse neuroblastoma cells through the hydrolysis of phosphatidic acid (PA) (Bisogno et al., 1997b). In both cases, the *sn*-1-selective DAG lipase catalyses DAG hydrolysis to 2-AG. Moreover, precursors other than DAGs, such as 1-lyso-phosphoglycerides (Ueda et al., 1993) can also generate 2-AG.

1.4.2 Inactivation of endocannabinoids

Anandamide and 2-AG can diffuse through the cell membrane quite rapidly (Di Marzo, 1999). However, recently, a selective, temperature-sensitive, saturable and Na^+ -independent "anandamide transporter" protein was described in several primary neuron cultures and cell lines (Hillard et al., 1997; Beltramo et al., 1997; Bisogno et al., 1997a). Interestingly, the "anandamide transporter" was recently shown to be significantly stimulated by nitric oxide donors, thus, indicating a putative regulatory pathway for the inactivation of endocannabinoids (Maccarrone et al., 1998; 2000) (Fig. 1.5). No evidence has been yet presented for a similar uptake mechanism for 2-AG. Once inside the cells, anandamide and 2-AG are degraded to arachidonic acid and ethanolamine (Fig. 1.5) and glycerol, respectively. In 1996, the complete cDNA encoding for an enzyme able to specifically hydrolyse anandamide and other fatty acid amides was cloned (Cravatt et al., 1996). This enzyme, generally referred to as fatty acid amide hydrolase (FAAH), is present in brain areas where high levels of CB1 are also present (Thomas et al., 1997; Tsou et al., 1998b; Marsicano & Lutz, unpublished observations). While FAAH is the major anandamide-degrading enzyme (Katayama et al., 1997), FAAH is also able to hydrolyse 2-AG (Goparaju et al., 1998), but evidence also exists that other hydrolases also contribute to 2-AG breakdown in the CNS (Bisogno et al., 1997b; Di Marzo et al., 1998; Goparaju et al., 1999). Interestingly, both the "anandamide transporter" and FAAH are able to transport and degrade, respectively, also other fatty acid amides different from anandamide, such as N-linoleoyl-ethanolamide (Maurelli et al., 1995; Maccarrone et al., 1998) or *cis*-9-octadecenoamide, also called oleamide (Cravatt et al., 1996). These and other similar compounds have no direct cannabinoid activity, but are often released from neurons in higher amounts than anandamide (Di Marzo et al., 1994). Considering that the "anandamide transporter" and FAAH are likely to be very important for the physiological inactivation of anandamide *in vivo* (Beltramo et al., 1997; Compton

& Martin, 1997), it is therefore possible that these naturally occurring compounds contribute to the elevation of the endogenous levels of anandamide and 2-AG, by competing at transport and degradation sites.

In conclusion, despite many still open questions regarding various aspects of the biology of cannabinoids, it is now accepted that the entity of endocannabinoids with their release, transport and degradation systems, and the CB1 receptor, with its intracellular signal transduction mechanisms, constitute a novel neuronal system that could be involved in several important functions of the CNS.

1.5 Proposed roles of the cannabinoid system in the CNS

As mentioned above (1.3 "CB1, the 'brain type' cannabinoid receptor"), the intracellular pathways triggered by CB1, together with the anatomical distribution of the receptor, can account for most of the effects induced by pharmacological treatment of animals with cannabinoids (Breivogel & Childers, 1998). However, the recent discovery of endocannabinoids and the partial elucidation of the mechanisms involved in their regulation, allowed the researchers to hypothesise some of the physiological and/or pathophysiological functions which the cannabinoid system might be involved in. Here, I will briefly summarise the present evidence regarding the roles of the endocannabinoid system in various CNS functions and some of the potential therapeutic applications.

1.5.1 Nociception

In mammals, Δ^9 -THC and synthetic CB1 agonists exert strong analgesic effects in several pain paradigms and through different administration routes, including local spinal and supraspinal applications (for recent reviews, see Martin and Lichtman, 1998; Chaperon & Thiébot, 1999). Exogenous cannabinoid analgesia appears to be partly connected with the opioid system (reviewed by Manzanares et al., 1999). Experiments using different opioid receptor antagonists administered either intrathecally (i.t.) or

intracerebroventricularly (i.c.v.) indicated that κ -opioid receptors and μ -opioid receptors are involved in the spinal and supraspinal analgesic effects of cannabinoids, respectively (Reche et al., 1996). When pharmacologically administered, endogenous cannabinoids, such as anandamide, are also able to induce analgesia (Stein et al., 1996; Compton & Martin, 1997; Adams et al., 1998). However, the opioid system does not appear to be involved in the analgesic effect of exogenously administered anandamide (Welch et al., 1995), thus, pointing to pharmacological differences between endocannabinoids and exogenous cannabinoid compounds. Recent studies have suggested that the endogenous cannabinoid system may modulate pain perception. Intrathecal administration of either the CB1 antagonist SR 141716A or antisense oligonucleotides complementary to CB1 mRNA, which resulted in a 60% decrease in CB1 binding sites, elicited significant hyperalgesia in rats (Richardson et al., 1998; Edsall et al., 1996). Analgesia induced by anandamide administration was reversed by N-methyl-D-aspartate receptor antagonists, thus, indicating the involvement of glutamate transmission in the analgesic effects of endogenous cannabinoids. Moreover, in a peripheral pain model, Calignano et al. (1998) showed that endogenous cannabinoids can produce analgesic effects also at CB1-like and CB2-like receptors located in the periphery, and, given the hyperalgesic properties of SR 141716A in the same tests, suggested a tonic activation of the endogenous cannabinoid system directed to "buffer" the noxious stimuli. Furthermore, recently, Walker et al. (1999) demonstrated that peripheral pain stimuli were able to induce a significant release of anandamide in the periaqueductal grey, a brain area mainly devoted to the processing of nociceptive information. Discrepant results were obtained in genetic approaches. In 1999, two CB1-deficient mouse lines were established by two different groups (Zimmer et al., 1999; Ledent et al., 1999). Unexpectedly, regarding pain perception, the mouse line generated by Zimmer and co-workers showed a marked hypoalgesia. On the other hand, Ledent et al. (1999) were not able to detect any changes in basal pain perception in

their mutant mice. Besides the reasons putatively at the basis of such discrepancy (discussed in Introduction of Chapter 4), both mouse lines did not show the exogenous cannabinoid-induced analgesia. Interestingly, in further studies, the mouse line generated by Ledent et al. (1999), despite showing a normal antinociceptive response to opioid administration, did not possess the so-called "stress-induced" analgesia, which is believed to depend on the endogenous opioid system (Valverde et al., 2000). Moreover, the mouse line from Zimmer et al. (1999) presented a significant increase in the expression of some endogenous opioids, such as dynorphin and enkephalin (Steiner et al., 1999). Although, as discussed in the Introduction of Chapter 4, these phenotypic aspects of CB1-mutant mice can be due to interactions between cannabinoid and opioid systems occurring at embryonic stages, nevertheless, such interaction might be reconsidered as an important point for the nociceptive functions of the endogenous cannabinoid system.

In rodents, a combination of morphine with Δ^9 -THC or CP 55,940 resulted in greater than additive antinociceptive effects in several pain models (Reche et al., 1996; Smith et al., 1998; Pugh et al., 1996). This suggests the use of cannabinoids as potential analgesic drugs that could be used in combination with other analgesics, with the advantage of reducing potentially negative side effects. Particularly interesting appears the possibility that the endocannabinoid system is involved in particular pathophysiological conditions, such as chronic and inflammatory neuropathic pain (Herzberg et al., 1997). This would imply that drugs able to modulate the activity of endogenous cannabinoids might be used in order to increase their "natural" analgesic effect. However, the direct participation of endocannabinoids to these pathological conditions has still to be confirmed and discrepant results have recently been presented (Bealieu et al., 2000; Piomelli et al., 2000; Di Marzo et al., 2000).

1.5.2 Locomotion

Exogenous cannabinoids profoundly affect locomotion in animals, in a dose-related biphasic manner. Large doses of natural and synthetic CB1 agonists (including anandamide) strongly reduce motor activity and can induce catalepsy, while low doses could stimulate motor activity (Smith et al., 1994; Sulcova et al., 1998; Rodriguez de Fonseca et al., 1998; Sañudo-Peña et al., 1999; Sakurai et al., 1985). The CB1 antagonist SR 141716A is able to reverse the locomotor inhibitory effects of CB1 agonists (Rinaldi-Carmona, 1994; Compton et al., 1996). When administered alone, the antagonist could induce hyperactivity in drug-naïve mice, however larger doses than those necessary to counteract the effects of cannabinoid agonists were required (Compton et al., 1996). As for nociception, discrepant results were gained in the CB1-deficient mice. Ledent et al. (1999) saw a modest stimulation of locomotor activity, while Zimmer et al. (1999) observed a severe hypomotility. CB1 is highly expressed in the basal ganglia, a brain region that plays a crucial role in motor-related processes, with a high density of CB1 protein in outflow nuclei, such as the entopeduncular nucleus (internal globus pallidus in primates), substantia nigra pars reticulata and external globus pallidus (Herkenham et al, 1990; Tsou et al., 1998a). Dorsolateral caudate putamen and subthalamic nucleus contain CB1 mRNA, thus indicating GABAergic striatonigral and glutamatergic subthalamonigral axons as the main CB1-containing subcellular structures (Mailleux & Vanderhaegen, 1992; Matsuda et al., 1993). A modulation of dopaminergic, GABAergic and glutamatergic transmission in these regions has been proposed for the cannabinoid-induced motor deficits (Glass et al., 1997b; Sañudo-Peña & Walker, 1997). Indeed, the potent synthetic CB1 agonist WIN 55,212-2 was shown to be able to reduce the spontaneous firing rate of neurons in the globus pallidus (Miller & Walker, 1996), and exogenously administered anandamide and CP 55,940 decreased the electrically-evoked release of dopamine in striatal slices (Cadogan et al., 1997). A cannabinoid-

mediated reduction of GABA uptake was shown in substantia nigra and globus pallidus (Romero et al., 1998; Maneuf et al., 1996).

In recent years, the endogenous cannabinoid system has been proposed to play an important role in locomotion, both in physiological and pathophysiological conditions (Rodriguez de Fonseca et al., 1998). Anandamide was found in microdialysates from the dorsal striatum of freely moving rats (Giuffrida et al., 1999). Its level was increased by depolarisation and was enhanced by stimulation of dopamine D2 receptors, whereas counteraction of its possible action at CB1 with SR 141716A enhanced the motor-stimulatory effect of the D2 agonist quinpirole. Therefore, the authors proposed that the endocannabinoid system may act as a feedback mechanism inhibiting dopamine-mediated stimulatory activity. Anandamide and 2-AG were also shown to be present at high levels in the substantia nigra and external layer of the globus pallidus (Di Marzo et al., 2000). Interestingly, in the same study, the authors revealed a net increase of pallidal 2-AG in reserpine-treated animals, a model of Parkinson's disease, whereas D1 or D2 receptor stimulation, respectively, in the same animals were able to decrease the levels of both endocannabinoids. Finally, full restoration of locomotion in the reserpine-treated animals was obtained by coadministration of the CB1 antagonist SR 141716A and the D2 agonist quinpirole, thus indicating a link between the endocannabinoid signalling in the globus pallidus and symptoms of Parkinson's disease. Indeed, clinical studies have shown that cannabinoid agonists can be used to reduce the levodopa-induced dyskinesia in Parkinson's disease (Muller-Vahl et al., 1999; reviewed in Sevcik & Masek, 2000). Also other neurological disorders connected with altered locomotor function have been proposed to involve a dysfunctional cannabinoid system. Therefore, cannabinoid-related therapy might be beneficial. As examples, in human brain derived from Huntington's chorea patients, specific CB1 binding appeared to be decreased in the basal ganglia at very early stages of the disease (Glass et al., 1993; Glass et al., 2000). Similar results

were obtained in a transgenic mouse model of the disease, with a net decrease of the CB1 mRNA levels in subsets of neurons of striatum, cortex and hippocampus before the onset of the signs of the disease (Denovan-Wright & Robertson, 2000). The Gilles de la Tourette syndrome has also been proposed as a target of cannabinoid therapy (Muller-Vahl et al., 1997). Of particular interest is a recent report concerning the ability of cannabinoids to control spasticity and tremor in a mouse model of multiple sclerosis (Baker et al., 2000). The authors showed not only that pharmacological treatment with CB1 agonists were able to drastically reduce these signs, but also that treatment of the animals with CB1 and CB2 antagonists were able to exacerbate them. Therefore, they concluded that the endogenous cannabinoid system may be tonically active in the control of tremor and spasticity in multiple sclerosis. This and others studies indicate that therapeutic uses of cannabinoids should rely more on the modulation of the endogenous cannabinoid system (by, for example, the use of drugs able to reduce the uptake or the degradation of endocannabinoids) rather than on the administration of exogenous cannabinoid agonists and antagonists (for recent reviews, see Di Marzo et al., 2000; Piomelli et al., 2000).

1.5.3 Learning and memory

Synthetic and natural cannabinoids are able to inhibit memory formation in animals as assessed by various paradigms and generally exhibit their actions at doses that do not decrease locomotor activity (Heyser et al., 1993; Nakamura et al., 1991 Ferrari et al., 1999). These effects are inhibited by the specific CB1 antagonist SR 141716A, thus indicating the involvement of the receptor. CB1 is highly expressed in brain areas that are considered as central elements for various kinds of memory formation, that are related to structures such as hippocampus, retrohippocampal areas (entorhinal and perirhinal cortex), amygdaloid nuclei and septal nuclei (Herkenham et al., 1990; Matsuda et al., 1993; Tsou et al., 1998a). CB1 is expressed in various subtypes of cells in these areas.

Both GABAergic interneurons and glutamatergic pyramidal cells express CB1 at various levels (Matsuda et al., 1993; Tsou et al., 1998a; Katona et al., 1999; Marsicano & Lutz, 1999; see also Chapter 2). Therefore, the mechanism of action of cannabinoids in these areas can be different depending on the cell types involved, but in most cases it appears to include a presynaptic inhibition of neurotransmitter release. In the hippocampus, cannabinoids were shown, to decrease the release of glutamate, acetylcholine, noradrenaline and GABA (Shen et al., 1996; Gifford et al., 1997; Schlicker et al., 1997; Katona et al., 1999). Such inhibitory effects on neurotransmitter release are considered to be the basis of the CB1-mediated blockade of long term potentiation (LTP) in hippocampal slices, an electrophysiological model of synaptic plasticity (Nowicky & Teyler, 1987; Terranova et al., 1995; Misner & Sullivan, 1999). Interestingly, a recent report by Nava et al. (2000a) showed that memory impairment caused by Δ^9 -THC and the associated reduction of hippocampal acetylcholine concentrations were inhibited not only by the CB1 antagonist SR 141716A, but also by the dopamine receptor D2 antagonist sulpiride. This would indicate that cannabinoid-induced memory impairment are mediated by a concomitant D2 and CB1 stimulation, thus pointing to an involvement of a cannabinoid dopamine cross-talk also in learning and memory processes. The endocannabinoids anandamide and 2-AG are also present in hippocampus and are able to inhibit LTP in a CB1-mediated manner (Stella et al., 1997; Felder et al., 1996; Terranova et al., 1995). In particular, 2-AG production appears to be enhanced by electrical stimulation of hippocampal slices (Stella et al., 1997). In early experiments, exogenously administered anandamide failed to impair memory processes (Crawley et al., 1993; Lichtman et al., 1995), likely due to the metabolic instability of the compound. Indeed, anandamide impaired memory in rats pre-treated with the protease inhibitor phenylmethylsulphonyl fluoride (Mallet & Beninger, 1998) and the metabolically stable analogue R-methanandamide impaired memory in rats (Brodkin and Moerschbaecher,

1997). The endogenous cannabinoid system has been proposed to be tonically stimulated during active forgetting processes in which less important information is deleted from memory storage (Hampson & Deadwyler, 1998; Collin et al., 1995). This notion is supported by several experiments. Treatment with the CB1 antagonist SR 141716A was shown to enhance social recognition (Terranova et al., 1996) and, more recently, to increase spatial memory in a delay version of the radial-arm maze task (Lichtman, 2000). Moreover, CB1-KO mice showed also enhanced memory in a paradigm of object recognition (Reibaud et al., 1999) and increased hippocampal LTP (Bohme et al., 2000). However, in contrast to the apparent memory enhancing effects of SR 141716A in some forms of memory, these results were not confirmed in a variety of different operant tasks (Brodkin & Moerschbaecher, 1997; Mallet and Beninger, 1998; Mansbach et al., 1996). These apparent discrepancies can indicate a tonic role of endocannabinoids in the modulation of some forms of memory and not in others. It is interesting to note that in both tasks where SR 141716A was shown to improve performances (Terranova et al., 1996; Lichtman, 2000), the animals had to retain the information for substantially longer periods of time than in the tasks where no improving effects were observed. This would indicate that the endocannabinoid tone is involved more in a long-lasting active forgetting than in the immediate erasing of "unwanted information".

The negative effects of cannabinoids on memory performance and the apparent enhancing effects of CB1 antagonists suggest cannabinoid-related treatment in pathologies with alterations of various forms of memory. As an example, the use of the CB1 antagonist SR 141716A has been proposed in the pathological conditions marked by memory deficits, as in traumatic brain injury or neurodegenerative disease, such as Alzheimer's disease (Lichtman, 2000).

1.5.4 Reward and motivational systems

Cannabis sativa derivatives have been known for millennia for their recreational and pleasure-inducing effects (Mechoulam, 1986). Among the many pharmacological effects produced by cannabinoid preparations is the dose-dependent "high", which is mainly due to the presence of Δ^9 -THC. On this basis, many studies in recent decades have attempted to clarify the mechanisms involved in these reward-inducing effects of cannabinoids by using animal models. In animals, motivational properties of drugs can be approached by three main behavioural procedures. 1) Intravenous self-administration (Goudie, 1991). 2) Drug-induced alterations of intracranial electrical self-stimulation, which occurs at a variety of electrode placements (the medial forebrain bundle being one of the more reward-relevant sites) (Stellar & Rice, 1989) 3) Place conditioning procedures, which are based on the principle that animals would learn to approach or avoid previously neutral environmental stimuli which have been paired with rewarding or aversive events, respectively (Carr et al., 1989). Numerous attempts to establish cannabinoids as reinforcers or self-administered drugs in naïve or drug-experienced rats or monkeys were unsuccessful (e.g. Leite & Carlini, 1974). Moreover, several studies gave evidence that synthetic and natural cannabinoids behave as negative reinforcers in rodents (McGregor et al., 1996; Parker & Gillies, 1995). However, in different experimental conditions, cannabinoids were shown to possess some reinforcing effect. As examples, Δ^9 -THC was able to reduce the current threshold intensity for intracranial self-stimulation in the medial forebrain bundle in some strains of rats (Lepore et al., 1996), was self-administered at low concentrations in rats, after previous food deprivation (Takahashi & Singer, 1979), and supported place preference in rats at doses that differed depending on the time interval between injections (Lepore et al., 1995). Self-administration of cannabinoids was more consistently observed in mice. WIN 55,212-2 was self-injected by CD1 mice at moderate concentrations and became aversive at higher

doses. Such effects were likely CB1-mediated, as they were abolished by SR 141716A and were absent in CB1-knock out mice obtained in the same CD1 genetic background (Martellotta et al., 1998; Ledent et al., 1999). Genetically determined strain differences could account for the discrepant results in drug preference, self-administration and intracranial self-stimulation obtained in rodents. Indeed, in the same experimental paradigm, Δ^9 -THC facilitated intracranial self-stimulation in the medial forebrain bundle at highest levels in Lewis rats, at half values in Sprague-Dawley rats, but had no effect in Fisher 344 rats (Lepore et al., 1996). However, cannabinoids were shown to increase responding for palatable drinking, such as beer, near-beer and sucrose solutions, in a CB1-dependent manner (Gallate et al., 1999) and to facilitate electrical self-stimulation in lateral hypothalamus, an area involved in feeding behaviour (Trojnar & Wise, 1991). These results would indicate that cannabinoids (although not consistently reinforcers *per se* and in some cases even producing aversion) may be active in facilitating the motivational effects of other positive reinforcers (Chaperon & Thiebot, 1999). This hypothesis is supported also by experiments in which the CB1 antagonist SR 141716A was shown to block the establishment of conditioned place preference generated by classical reinforcers, such as cocaine, morphine and food (Chaperon & Thiebot, 1999). Moreover, morphine self-administration was highly reduced in CB1-knock out mice (Ledent et al., 1999). SR 141716A also reduced motivation for sucrose solution, beer and alcohol consumption (Gallate & McGregor, 1999; Arnone et al., 1997; Colombo et al., 1998). Taken together, these results suggest that the endocannabinoid system is likely to be required for the perception of the motivational value of positive reinforcers, even if these reinforcers do not directly interact with the cannabinoid system itself (Chaperon & Thiebot, 1999).

From the anatomo-functional point of view, the main circuits related to reward appear to consist of a synaptically interconnected series of neuronal tracts closely

associated with the medial forebrain bundle (reviewed in Gardner, 1997). Three stages can be individuated in the neuronal transmission connected with the reward circuits (Gardner & Vogel, 1998). 1) From various external reinforcing stimuli, a "first stage" of reward circuits originate diffusely in the ventral anterior forebrain, from different anterior bed nuclei of the medial forebrain bundle. Among these, CB1 mRNA is present in the anterior lateral hypothalamus, in the horizontal limb of the diagonal band of Broca, in the lateral preoptic area, in the magnocellular preoptic nucleus and in the olfactory tubercle (Matsuda et al., 1993). From these and other nuclei of the medial forebrain bundle, projecting axons run posteriorly within the medial forebrain bundle and synapse on dopaminergic cells in the ventral tegmental area. These projections are believed to encode for information critical to the set point of hedonic tone (Gardner & Vogel, 1998). 2) Dopaminergic neurons project anteriorly, always within the medial forebrain bundle to the nucleus accumbens and constitute the "second stage" of reward-related transmission. It is on this "second stage" dopaminergic convergence that addictive and abusable substances appear to increase brain reward functions and produce the pleasurable and euphoric effects at the basis of addiction (Gardner & Vogel, 1998; Kornetsky & Duvauchelle, 1994). 3) "Third stage" neurons appear to carry reward-relevant information from nucleus accumbens to the ventral pallidum, using as primary neurotransmitter the endogenous opioid peptide enkephalin (Gardner & Vogel, 1998). In order to complicate the picture, a number of additional circuits coming from several distinct anatomical regions such as prefrontal cortex, amygdala, locus ceruleus, raphe nucleus and others, synapse onto the above neuronal "stages", apparently to regulate and modulate the overall set point of hedonic tone (Gardner & Vogel, 1998). In this complex situation, cannabinoids acting at CB1 are likely to interact at different stages with reward-related circuits. However, the mesolimbic dopaminergic system is considered as the common neuronal substrate for the motivational and rewarding effects of drugs and

for related physiological functions of the brain. Therefore, cannabinoids have been extensively studied in regard of their influence on this system. Ventro tegmental dopaminergic neurons are likely to be devoid of CB1, but anatomical and functional interactions between CB1 and dopamine receptors were described in dorsolateral striatum (e.g. Glass & Felder, 1997; Giuffrida et al., 1999; Hermann, Marsicano & Lutz, *in preparation*). Even in absence of direct experimental evidence, given the fact that nucleus accumbens is coextensive with the ventral striatum and that there is extensive homology between the afferents, efferents and internal neuroanatomical organisation of the nucleus accumbens and those of the dorsal striatum (Gerfen, 1988; 1993), it can be assumed that the anatomofunctional interactions between dopaminergic and cannabinoid systems found in dorsolateral striatum may also hold at some extent in the nucleus accumbens (Gardner & Vogel, 1998). However, Δ^9 -THC has been reported to enhance dopamine outflow in nucleus accumbens and medial prefrontal cortex of rats (Chen et al., 1990a; 1990b). More recently, Δ^9 -THC and WIN 55,212-2 were shown to induce dopamine release selectively in the shell, but not in the core, of nucleus accumbens. Such an effect was completely prevented by SR 141716A (Tanda et al., 1997). These cannabinoid actions were also strongly reduced by the aspecific opioid receptor antagonist naloxone and by the μ_1 opioid specific antagonist naloxonazine, thus indicating that CB1 stimulation may facilitate mesolimbic dopamine transmission by activating the endogenous opioid system (Tanda et al., 1997; Chen et al., 1990a). *In vivo* electrophysiological studies have also shown that cannabinoids acting at CB1 are able to activate mesolimbic and meso-prefrontal dopaminergic neurons (Diana et al., 1998; Gessa et al., 1998; French, 1997). On the other hand, SR 141716A did not change the spontaneous firing rate of mesolimbic dopaminergic cells, thus presumably excluding that these neurons are under the control of a tonically activated cannabinoid system (Gessa et al., 1998).

In conclusion, despite the fact that behavioural data are at some extent contradictory in revealing positive reinforcing properties of cannabinoids *per se*, an involvement of the cannabinoid system exists in many aspects of reward-related brain functions, mostly mediated by interactions with the dopaminergic and the opioid systems. In this respect, CB1 antagonists treatment has been proposed for the management of opioid addiction (Ledent et al., 1999). On the other hand, cannabinoids or drugs aimed to enhance endogenous cannabinoid tone might be useful in the treatment of dysphoric states (Gardner & Vogel, 1998).

1.5.5 Neuroprotection

Natural and synthetic exogenous cannabinoids were shown to exert neuroprotective effects in several *in vivo* and *in vitro* neurotoxicity models (Hampson et al., 1998a; Nagayama et al., 1999; Sinor et al., 2000). Also endocannabinoids and related compounds have been proposed to exert neuroprotective activity (Di Marzo et al., 1998). Anandamide is able to inhibit NMDA-receptor-mediated Ca^{2+} influx (Hampson et al., 1998b) and has been shown to be released following glutamate stimulation or cell injury (Schmid et al., 1996; Cadas et al., 1996; Hansen et al., 1998). However, the exact mechanism(s) of these neuroprotective effects have not yet been clarified. "Classical" and "non-classical" cannabinoids such as Δ^9 -THC and CP 55,940 contain a phenolic ring (Fig. 1.4) which has been proposed as an important lead structure for protection against oxidative stress, regardless of any specific receptor-mediated action (Moosmann & Behl, 1999). Therefore, the presence of a phenolic ring in many exogenous cannabinoids could account for their neuroprotective effects. Indeed, Δ^9 -THC, but also another constituent of *C. Sativa*, cannabidiol, which has a very low affinity for CB1, were both shown to act as neuroprotective antioxidant (Hampson et al., 1998a). However, other mechanisms appear to be also involved. Aminoalkylindole compounds, such as WIN 55,212-2, and endocannabinoids do not contain the phenolic ring and, indeed, do not show antioxidant

properties (Marsicano, Moosmann, Behl & Lutz, *in preparation*; see also Chapter 5). However, WIN 55,212-2 was shown to be a potent neuroprotectant in a rat model of global and focal ischaemia (Nagayama et al., 1999). Interestingly, in the same report, the neuroprotective action of WIN 55,212-2 appeared to be CB1-mediated *in vivo*, but is CB1-independent *in vitro*, thus indicating differences in the mechanisms of action in the whole animal or in isolated neuronal cultures. Recently, endocannabinoids were also shown to protect cortical neurons from *in vitro* O₂ deprivation, in a CB1- and CB2-independent way (Sinor et al., 2000). Moreover, palmitoylethanolamide, a non-CB1-binding eicosanoid member of the same family of anandamide (Di Marzo et al., 1998), was shown to protect cerebellar granule cells from glutamate toxicity (Skaper et al., 1996). Finally, CB1 expression was recently shown to be increased in experimental stroke (Jin et al., 2000), thus indicating a likely involvement of CB1-mediated endogenous cannabinoid signalling pathways in self-protecting mechanisms of the brain. Taken together, these data suggest complex mechanisms underlying the neuroprotective effects of cannabinoids and the putative neuroprotective role of the endogenous cannabinoid system, ranging from chemical antioxidant properties of "classical" and "non-classical" cannabinoids to the receptor-mediated effects of aminoalkylindoles and endocannabinoids.

Whatever the underlying mechanism(s) are, these properties of exogenous and endogenous cannabinoids have been considered as promising in respect of possible therapeutic applications. Regulation of glutamate transmission, antioxidant properties and inhibition of Ca²⁺ permeability can constitute a "cocktail" of positive effects in neurodegenerative pathologies. In this context, cannabinoids or drugs aimed to increase the endocannabinoid tone might emerge as potentially very useful therapeutic agents in neurotoxic diseases, such as brain ischaemia or Alzheimer's disease (Piomelli et al., 2000).

1.5.6 Other proposed central roles of the cannabinoid system

Besides the roles mentioned above, exogenous and endogenous exert also other effects in animals and humans.

Natural and synthetic cannabinoids, as well as endocannabinoids produce a typical decrease of body temperature in animals (Pertwee, 1997). This effect is probably due to the presence of cannabinoid receptors in hypothalamic and caudal brainstem areas (Breivogel & Childers, 1998). Interestingly, recent results have shown that hypothermic effects induced by Δ^9 -THC are mediated by a coactivation of CB1 and dopamine D2-like receptors (Nava et al., 2000b), underlying the importance of a functional cross-talk between cannabinoid and dopaminergic systems. An active cannabinoid tone was never shown to exist in the control of basal temperature, either by CB1 antagonist treatment (Compton et al., 1996) nor in CB1-knock out mice (Zimmer et al., 1999; Ledent et al., 1999). However, given the facts that endocannabinoids are released in cellular stress conditions and that the cannabinoid system has been proposed to play a role in immune system and in inflammation (reviewed by Di Marzo et al., 1998), a role of the cannabinoid system in the modulation of body temperature in stress conditions might be hypothesized.

The central endogenous cannabinoid system has also been proposed to have a role in sleep (Murillo-Rodríguez et al., 1998). The mechanism through which anandamide and other cannabinoids are able to induce sedation have not yet been clearly understood. However, very interestingly, the hypnotic actions of oleamide, a non-cannabimimetic fatty acid amide that accumulates in cerebrospinal fluids under conditions of sleep deprivation and that induces sleep in animals (Boger et al., 1998), were shown to be blocked by the CB1 antagonist SR 141716A (Mendelson & Basile, 1999), thus indicating a central role of the cannabinoid system in sleep-inducing mechanisms.

Appetite stimulation, relief of anxiety and sedation are three of the typical effects

described by *Cannabis sativa* extract users and have been correlated with the presence of cannabinoid receptors in the hypothalamus and limbic system (Di Marzo et al., 1998). The endogenous cannabinoid system has been proposed to play a tonic modulatory role in the sucrose and ethanol intake (Arnone et al., 1997), in the inhibition of anxiety-like responses (Navarro et al., 1997) and in the decrease of arousal (Santucci et al., 1996) in rodents. Taken together, the main central effects of cannabinoids and the proposed roles of the endocannabinoid system could be related to a general stress-recovery system (Di Marzo et al., 1998). "Feel less pain, control your movement, relax, eat, forget, sleep and protect" might be some of the signals that are mediated by the activation of the endogenous cannabinoid system.

1.6 Aims of the Thesis

Exploring some of the physiological and pharmacological aspects of the cannabinoid system was the major aim of this thesis. Several different methodological means were applied to investigate the biology of the mouse cannabinoid system and of the "brain type" cannabinoid receptor CB1 in particular.

In Chapter 2 and partly in Chapter 3, single and double *in situ* hybridization experiments were performed to determine the CB1-expressing neuronal populations in the forebrain at the cellular level that are expressing the receptor. There were two purposes for these studies. First, to better understand the mechanisms through which the cannabinoid system might modulate neural activity in the brain; second, to describe genes that are coexpressed with CB1 in the adult mouse brain in the light of defining which Cre recombinase-expressing transgenic mouse lines could be used to generate "conditional" mouse mutants (see "Discussion" of Chapter 4). Both aims were reached, with a detailed description of the expression pattern of CB1 at cellular level and the definition of two genes, cholecystinin and glutamic acid decarboxylase, whose regulatory sequences appear optimal candidates for spatially restricted expression of Cre recombinase in transgenic mouse lines.

Neuroanatomical experiments constitute a good tool to get a deeper insight into possible physiological functions of a protein. However, it was also my interest to investigate some more functional aspects of the cannabinoid system. From the results described in Chapter 2, it was possible to hypothesise an involvement of CB1 in the modulation of GABAergic and glutamatergic neurons. It is known that glutamatergic transmission is strictly connected with the nitric oxide pathway. Therefore, the availability of mutant mice for the gene nNOS (neuronal nitric oxide synthase) represented a good opportunity to explore in details one of the putative functional connections of the cannabinoid system with other neurotransmitter systems. With this

aim, in Chapter 3, the functional cross-talk between cannabinoid system and nitric oxide (NO) pathway was analysed. nNOS-knock out mice were behaviourally assayed for cannabinoid-induced pharmacological effects. Moreover, nNOS mutants were characterised for the expression of CB1 in selected brain areas by *in situ* hybridization and coexpression between CB1 and nNOS was analysed by double *in situ* hybridization in wild type mice. Results indicate that nNOS is necessary for some behavioural effects of Δ^9 -THC, that nNOS mutant mice express lower levels of CB1 mRNA in selected brain areas and that CB1 and nNOS are coexpressed in some regions of mouse forebrain.

Chapter 4 constituted the central part of this thesis. It describes a sophisticated genetic approach to investigate very specifically and precisely the physiological functions of CB1. The most important steps for the generation of a "conditional" CB1-mutant mouse line are presented, with the obtaining of the "Floxed CB1" line and of the cannabinoid receptor null mice ("CBN" line). Several techniques were used for the preparation of this Chapter, ranging from molecular biology, to cell culture and mouse breeding methods. The "Floxed CB1" line will represent a powerful tool during the next years for the genetic analysis of the functions of CB1, whereas the "CBN" line represents an interesting model for the investigations on the activity of CB1 at cellular level.

One example of these investigations is described in Chapter 5. In the last Chapter of my thesis, I analysed some aspects of the neuroprotective properties of cannabinoids, by the use of *in vitro* neurotoxicity assays. Cannabinoids were shown to possess neuroprotective potentialities, but the exact mechanism(s) of these effects is still unclear. Therefore, several cannabinoids were tested in *in vitro* neurotoxicity assays and the involvement of CB1 in cannabinoid-mediated neuroprotection was analysed. Cell lines were permanently transfected with CB1 and neuroprotective effects of various cannabinoids were tested on such cell clones in comparison to the parental wild type cell clones. Moreover, primary cerebellar granule cells derived from wild type mice and from

CB1-knock out mice (CBN mouse line, as generated in Chapter 4) were also used to determine the role of CB1 in cannabinoid-induced *in vitro* neuroprotection. Results seem to indicate that CB1 is not necessary for cannabinoid-mediated neuroprotection *in vitro*.

In conclusion, the overall strategy of the Thesis tried to cover several aspects of physiological roles of the cannabinoid system and of CB1 in the mouse brain. Neuroanatomical and neurochemical results, as obtained in Chapter 2 and 3, represented the rationale for a functional analysis (cross-talk between cannabinoids and nitric oxide pathway in Chapter 3) and for the future spatial deletion of CB1 (see the Discussion of Chapter 4). The generation of CB1-mutant mice and in particular the "conditional" deletion of CB1 (Chapter 4) will represent a powerful tool for understanding in details the physiological roles of the cannabinoid system in mouse physiology and behaviour. Moreover, null CB1-deficient animals were used in this Thesis as a source of neurons to analyse the roles of CB1 in *in vitro* neuroprotection of cannabinoids (Chapter 5). Although much work is still to be done in the future, the work described in this Thesis led to new insights into the roles of CB1 and the cannabinoid system.

Chapter 2

Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain

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2.1 Introduction

The characteristic behavioural effects of cannabinoids are mediated by CB1, the "brain type" cannabinoid receptor, a seven transmembrane G-protein-coupled receptor (Matsuda et al., 1990). Biochemical studies showed that $G_{i/o}$ proteins transduce the cannabimimetic inhibition of adenylate cyclase (Howlett, 1995). CB1 causes a cAMP-independent inhibition of N- and Q-type voltage-dependent Ca^{2+} channels (Mackie & Hille, 1992; Felder et al., 1995) and a cAMP-dependent stimulation of inwardly rectifying K^+ channels (Deadwyler et al., 1993; Mackie et al., 1995). These signal transduction pathways can explain some effects of cannabinoids in the central nervous system, such as inhibition of acetylcholine, noradrenaline and glutamate release, e.g. in hippocampus (Gifford & Ashby, 1996; Schlicker et al., 1997; Shen et al., 1996). CB1 is also coupled to the activation of adenylate cyclase in striatal neurons upon simultaneous stimulation of the dopamine receptor D2 (Glass & Felder, 1997), possibly due to differential regulation of adenylate cyclase isozymes by CB1 (Rhee et al., 1998). Endogenous ligands for CB1 have been discovered (Devane et al., 1992; Stella et al., 1997), sharing many of the properties of plant derived and synthetic cannabinoids. Therefore a cannabinoid system has been proposed to play an important neuromodulatory role in brain physiology (for review see Di Marzo et al., 1998).

Several pharmacological effects of cannabinoids (e.g. decrease of learning behaviour) are thought to be mediated by CB1 expressed in forebrain structures. *In situ*

hybridization (ISH) and immunohistological studies described CB1 expression in the rat forebrain in several cortical areas and in several non-cortical regions, such as striatum and some thalamic and hypothalamic nuclei (Mailleux & Vanderhaegen, 1992; Matsuda et al., 1993; Tsou et al., 1998a; Pettit et al., 1998). The pattern of CB1 expression in the mouse forebrain, however, has not yet been described in detail.

Determination of CB1-positive neurons at a single cell resolution is a prerequisite for better understanding of the physiological role of the cannabinoid system in mouse. Based on morphological features, CB1-expressing neurons in hippocampus and other cortical regions have been proposed to be GABAergic (Matsuda et al., 1993; Tsou et al., 1998a), but conclusive evidence for this notion is still missing. GABAergic neurons express GABA and glutamic acid decarboxylase (GAD), and are classified according to criteria such as morphology, orientation of afferent and efferent fibres, type of innervating neurons and presence of distinct calcium binding proteins and neuropeptides (for review see Freund & Buszáki, 1996). Morphological criteria are difficult to be evaluated in ISH experiments, but detection of coexpression of CB1 with various markers can very well be accomplished using double ISH technique, as transcripts are generally localized in the soma of the cells. Thus, in this study, it was investigated which CB1-expressing cells are GABAergic and whether CB1 is coexpressed with other neuronal markers.

Recent immunohistochemical studies in rats suggest that a fraction of CB1-positive cells in the hippocampus has morphological features of basket cells, which are also called perisomatic inhibitory cells (Tsou et al., 1998a). Typically, basket cells are forming "basket" nets around principal cell bodies. From the neurochemical point of view, basket cells are GABAergic neurons that can further be divided into two distinct subpopulations: (1) basket cells containing the calcium binding protein parvalbumin (PV), and (2) basket cells containing the neuropeptide cholecystokinin (CCK) (Freund &

Buszáki, 1996; Soriano et al., 1990; Gulyás et al., 1991). In addition, Tsou et al. (1998a) described CB1-positive neurons possessing fibers with orientations different from typical basket cells. Thus, CB1-expressing cells might belong to an additional subpopulation of hippocampal GABAergic neurons. Two putative candidates for these CB1-containing non-basket cells are calbindin D28k (C28)-positive mid-proximal dendritic inhibitory cells (Gulyás & Freund, 1996) and calretinin (CRT)-positive interneurons that are specialized in innervating other interneurons (Gulyás et al., 1996). Therefore, double ISH experiments were performed on hippocampus, but also on other cortical and some selected non-cortical areas of the mouse forebrain, using radioactive riboprobes for GAD 65, CCK, C28, PV and CRT, respectively, together with a non-radioactive CB1 riboprobe.

2.2 Materials and methods

2.2.1 Tissue preparation

Adult mice (3-5 months old; FVB/N and CD1 strains) were killed by cervical dislocation. Brains were removed, snap-frozen on dry-ice and stored at -80°C. After removing from -80°C, brains were mounted on Tissue Tek (Polysciences, Warrington, PA, U.S.A.), and 14-μm thick coronal sections were cut from forebrain on a cryostat Microtome HM560 (Microm, Germany). Sections were mounted onto frozen SuperFrost/Plus slides (Fisher Scientific, Ingolstadt, Germany), dried on a 42°C-warming plate and stored at -20°C until used.

2.2.2 Synthesis of probes

Both radioactive (³⁵S) and non-radioactive (fluorescein isothiocyanate, FITC) labeled riboprobes were used. Probes were generated by RT-PCR from cDNA derived from total mouse brain RNA. For each probe, GenBank accession number, length and sequence of the primers are listed below; nucleotide positions are identical to those used in deposited

sequences in GenBank: CB1, accession number U22948, 1530 base pairs (bp) from 152 to 1682 (forward primer 5'-GTT GAG CCT GGC CTA ATC AAA, reverse primer 5'-GTT GAC CGA ACC TCT GTT TTC); GAD 65, accession number D42051, 1041 bp from 1055 to 2096 (forward primer 5'-GGC GAT GGA ATC TTT TCT CCT, reverse primer 5'-CGA GGC GTT CGA TTT CTT CAA); PV, accession numbers X54613 and X67141, 802 bp from 11 of accession number X54613 to 510 of accession number X67141 (forward primer 5'-CAG CGC TGA GGA CAT CAA GAA, reverse primer 5'-GAT CTA GCT AGT CCT GAA GGA); CRT, accession number X73985, 1023 bp from 35 to 1058 (forward primer 5'-CCG ACC GAA GAG AAT TTC CTT, reverse primer 5'-GGG AAG CCA AAG AGA AAA GGA); C28, accession number M21531, 1016 bp from 166 to 1182 (forward primer 5'-GAG ATC TGG CTT CAT TTC GAC, reverse primer 5'-GAT GAA CAC TTG GAT TTC CC); CCK, accession number X59520 and X59522, 411 bp from 188 of accession number X59520 to 151 of accession number X59522 (forward primer 5'-ACT TAG CTG GAC TGC AGC TT, reverse primer 5'-GGA CTA CGA ATA CCC ATC GTA). PCR products were cloned into pBluescript KS⁺ (Stratagene, CA, U.S.A.) and used as templates for riboprobe synthesis. Identity of all fragments was checked by sequencing. Linearized template DNA was phenol-extracted, precipitated, resuspended in DEPC-treated H₂O at a concentration of 1 µg/µl, and stored at -20°C. For ³⁵S-labeled riboprobes, *in vitro* transcription was carried out for 3 hours at 37°C in a total volume of 30 µl containing 1.5 µg of linearized DNA, 1x transcription buffer, 1 mM of rATP/rCTP/rGTP each, 16.7 mM DTT, 40 units RNasin (Promega, U.S.A., WI), 10 µl of ³⁵S-thio-rUTP (NEN, U.S.A., MA; 1250 Ci/mmol) and 30 units of T7 or T3 RNA polymerase (Roche Molecular Diagnostics, Germany). For FITC-labeled riboprobes, *in vitro* transcription was carried out for 3 hours at 37°C in a total volume of 50 µl containing 1.5 µg of linearized DNA, 1x transcription buffer, 0.35 mM FITC-rUTP (Roche Molecular Diagnostics, Germany), 0.65 mM rUTP, 1 mM of rATP/rCTP/rGTP each, 80 units RNasin (Promega, WI, U.S.A.) and 100 units of T3 or T7

RNA polymerase. Reactions were treated with 20 units of RNase-free DNaseI (Roche Molecular Diagnostics, Germany) for 15 min at 37°C, and labeled probes were purified by ammonium acetate precipitation. Restriction enzymes (New England Biolabs, MA, U.S.A.) used for linearization and RNA polymerases used for each probe were as follows: CB1 sense, PstI, T7; CB1 antisense, BamHI, T3; GAD 65 sense, EcoRI, T7; GAD 65 antisense, BamHI, T3; PV sense, BamHI, T3; PV antisense, HindIII, T7; CRT sense, EcoRI, T7; CRT antisense, BamHI, T3; C28 sense, EcoRV, T7; C28 antisense, BamHI, T3; CCK sense, BamHI, T3; CCK antisense, EcoRI, T7. Using these probes in ISH experiments, sense controls did not give any detectable signals, and antisense probes gave distribution patterns identical to those already published in rat or mouse (data not shown).

2.2.3 *In situ* hybridization

Slides were warmed-up for 30 min at RT, fixed in ice-cold 4% paraformaldehyde in PBS (PBS contains 136 mM NaCl, 2.7 mM KCl, 10 mM, Na₂HPO₄, 1.8 mM KH₂PO₄, pH 7.4), rinsed three times in PBS, incubated for 10 min in 0.1 M triethanolamine-HCl (pH 8.0) to which 0.63 ml of acetic anhydride were added dropwise, rinsed twice in 2x SSC (1x SSC contains 150 mM NaCl, 15 mM Na₃ citrate, pH 7.4), dehydrated in graded series of ethanol, delipidized in chloroform for 5 min, rinsed in 100% and 95% ethanol and air-dried. Hybridization was carried out overnight at 64°C in 90 µl of hybridization buffer containing ³⁵S-labeled riboprobe (35,000 to 70,000 cpm/µl) and/or FITC-labeled riboprobe (1 µg/ml). Hybridization buffer consisted of 50% formamide, 20 mM Tris-HCl pH 8.0, 0.3 M NaCl, 5 mM EDTA pH 8.0, 10% dextran sulphate (D8906, Sigma, Germany), 0.02% Ficoll 400 (F2637, Sigma, Germany), 0.02% polyvinylpyrrolidone (MW 40,000, PVP40, Sigma, Germany), 0.02% bovine serum albumin (BSA; A6793, Sigma, Germany), 0.5 mg/ml tRNA (Roche Molecular Diagnostics, Germany), 0.2 mg/ml fragmented herring sperm DNA and 200 mM DTT.

After incubation in humid chamber, slides were rinsed four times for 5 min each in 4x

SSC at room temperature (RT), incubated 30 min at 37°C in 20 µg/ml of RNaseA in 0.5 M NaCl, 10 mM Tris-HCl pH 8.0, 5 mM EDTA, rinsed at RT in decreasing concentrations of SSC (1x, 0.5x and 0.1x SSC) containing 1 mM DTT, washed twice for 30 min each at high stringency in 0.1x SSC/1mM DTT at 64°C and washed twice for 10 min at RT in 0.1x SSC.

At this point, ³⁵S-labeled slides were dehydrated in graded ethanol series, air-dried and exposed to Biomax MR film (Kodak, Germany). On the next day, slides were dipped in photographic emulsion (NTB-2 from Kodak, diluted 1:1 in distilled water). After exposure for 5 to 20 days at 4°C, slides were developed for 3 min (D-19, Kodak, Germany), fixed for 6 min (Kodak fixer), rinsed for 30 min in tap water and air-dried. Slides were mounted in DPX (BDH, England).

For non-radioactive (FITC) and double-labeled (³⁵S/FITC) ISH experiments, slides were soaked after the last 0.1x SSC wash at RT (see above) in buffer 1 (100 mM maleic acid pH 7.5, 150 mM NaCl) for 1 min at RT, blocked in buffer 2 (1% Blocking Reagent in buffer 1, Roche Molecular Diagnostics, Germany) for 30 min at RT, incubated with alkaline phosphatase-conjugated anti-FITC antibody (Roche Molecular Diagnostics, Germany) diluted 1/3000 in buffer 2 for 2 hours at RT, washed 2x in buffer 1 for 15 min at RT, washed 2x in buffer 3 (100 mM Tris-HCl pH 9.5, 100 mM NaCl, 50 mM MgCl₂) for 2 min at RT. Chromogenic reaction was carried out with Vector Red kit (Vector Laboratories, U.S.A., CA) at RT for 24-36 hours with 2 changes of staining solution. Reaction was stopped by a 10-min incubation in 0.1 M Tris-HCl pH 8.2, followed by 10 min in 2.5% glutaraldehyde in PBS and four washes for 15 min in 0.1x SSC. In double ISH experiments, slides were treated at this point like slides from radioactive ISH, with dehydration, dipping and developing. At the end, slides were counterstained for 10 sec in 0.1% aqueous toluidin blue solution, rinsed 2x for 10 sec in tap water, destained for 45 sec in 70% ethanol (containing 1 drop of 100% acetic acid per 100 ml), rinsed for 45 sec in 100% ethanol and air dried. Slides from double ISH were mounted in Kaiser's Gelatin (BDH, U.K.).

2.2.4 Numerical evaluation of coexpression

As CB1 is expressed at various levels, cells stained with FITC-labeled riboprobe were classified according to the following criteria. Cells expressing CB1 at high levels (termed: high CB1-expressing cells) were considered those showing a round-shaped and intense red staining surrounding the nucleus or even covering the entire nucleus (e.g. see Fig. 2.2D,F). Cells expressing CB1 at low levels (termed: low CB1-expressing cells) were defined as cells clearly stained above background levels and in a discontinuous shape and/or at uniform and low intensity of staining (e.g. see Fig. 2.2D,F). Principal cells in CA1/CA3 regions of hippocampus displayed low and uniform levels of expression, with a slightly stronger intensity in CA3. Concerning numerical evaluation of the double ISH experiments, these principal cells were not included. Sections were analysed on a Leica DMRB microscope. All CB1-positive cells were checked for coexpression with the following markers: GAD 65, CCK, PV, CRT or C28, respectively. Cells were evaluated and classified as high CB1-expressing cells or as low CB1-expressing cells with or without coexpression of above markers, respectively. In hippocampus, CCK- and GAD 65-positive cells were also evaluated for coexpression with CB1.

2.3 Results

2.3.1 CB1 expression in the forebrain

In hippocampus (Fig. 2.1D; Fig. 2.2A,B), both low and high CB1-expressing cells were detected. Pyramidal cells (Py) express low levels of CB1, while cells with intensity of signal ranging from low to very high were observed in all layers of hippocampus, mostly in the subgranular layer of dentate gyrus (SGr) (see also in Fig. 2.3C) and the lacunosum-molecular layer of CA1 and CA3 regions (LMol) (Fig. 2.2A). Neocortex showed the presence of scattered low and high expressing cells, located primarily in layers II-III and V-VI (Fig. 2.1D, 2.2A). Few low CB1-expressing cells are scattered in layers I and IV. CB1-positive cells are more abundant in the primary and secondary motor (M1/M2) and sensory areas (S1FL/HL) (Fig. 2.1C,D). CB1 is also highly expressed in olfactory areas (Fig. 2.1A). Low CB1-expressing cells are distributed uniformly in the entire anterior olfactory nucleus (AO) with the presence of many scattered cells expressing high levels of mRNA (data not shown). Rather high numbers of CB1-positive cells (both low and high CB1-expressing cells) were observed also in piriform cortex (Pir) and olfactory tubercle (Tu) (Fig. 2.1B,C). In amygdaloid region, not all nuclei showed equal intensities of expression. Both anterior (BLA) (Fig. 2.1D; Fig. 2.2C,D) and posterior parts of basolateral amygdaloid nucleus (data not shown) contain a very high number of low CB1-expressing cells uniformly distributed, and a rather high number of scattered high CB1-expressing cells. Basomedial amygdaloid nucleus (BMA) showed a similar pattern of CB1 expression (Fig. 2.1D), while other amygdaloid nuclei contain lower levels of expression (data not shown). Entorhinal and perirhinal cortical areas (Ent) showed a very high number of both low and high CB1-expressing cells (Fig. 2.1C,D; Fig. 2.2E,F). Non-cortical areas such as striatum (dorsolateral caudate putamen, Cpu, and globus pallidus, GP) and hypothalamus (ventromedial, VMH, and anterior hypothalamic nucleus) showed the presence of low CB1-expressing cells that are uniformly distributed at high cell density (Fig. 2.1D). Expression levels in other hypothalamic areas and

in thalamus are relatively low as compared to other forebrain regions. Rather strong hybridization signals were detected in the septal region (lateral, Ls, and medial septum, Ms, and vertical and horizontal nuclei of the diagonal band, VDB) (Fig. 2.1B).

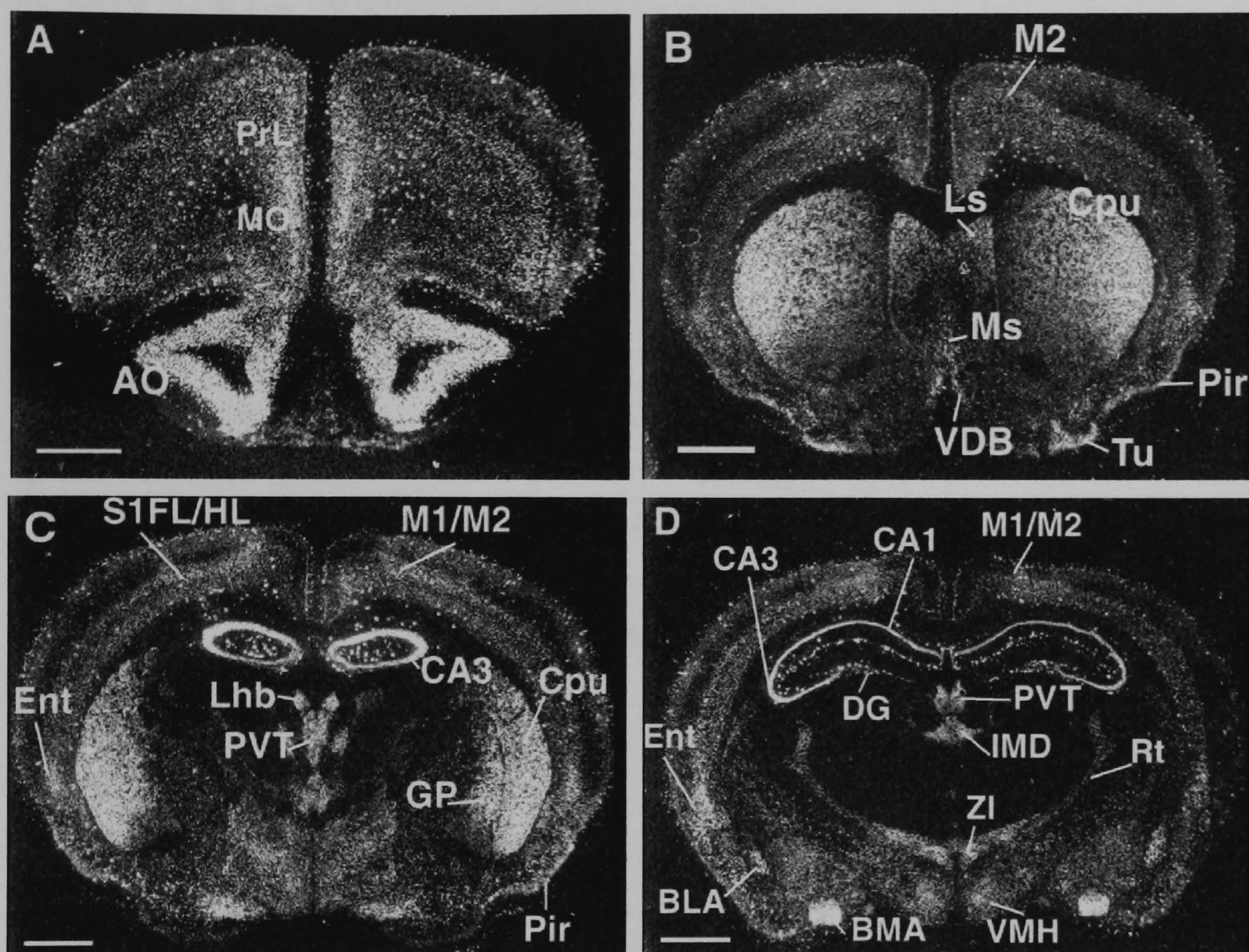


Fig. 2.1. Dark-field micrographs of coronal sections from adult mouse forebrain showing the distribution of CB1 mRNA, as detected by ISH with a ^{35}S -radiolabeled riboprobe for CB1. Note the high levels of CB1 expression in anterior olfactory nucleus (A), neocortex (A-D), dorso-lateral caudate putamen (B,C), hippocampus (C,D), entorhinal/perirhinal cortex area (C,D), basolateral and basomedial amygdaloid nuclei (D). Scale bars: 1 mm.

Rather high levels of expression were also observed in hypothalamic regions, such as medial and lateral preoptic nucleus, magnocellular preoptic nucleus and hypothalamic nucleus (not shown). More caudally, the premammillary nucleus showed low and uniform levels of expression (not shown). Scattered, low CB1-expressing cells were observed in the lateral hypothalamus (Fig. 2.1D). Weak signals were also observed in thalamic regions, such as the paraventricular thalamic nucleus (PVT), lateral habenula (Lhb), reticular thalamic nucleus (Rt) and zona incerta (ZI) (Fig. 2.1C,D). In summary, the pattern of CB1 expression in

mouse was found to be very similar, if not identical, to that observed in rats (Matsuda et al., 1993). Distribution, number and intensities of ISH signals generated with radioactive and non-radioactive riboprobes specific for CB1 were then compared. Representative examples are shown in Fig 2.2. No differences were detected between these two techniques, and, thus, non-radioactive riboprobes for CB1 were further used for double ISH experiments.

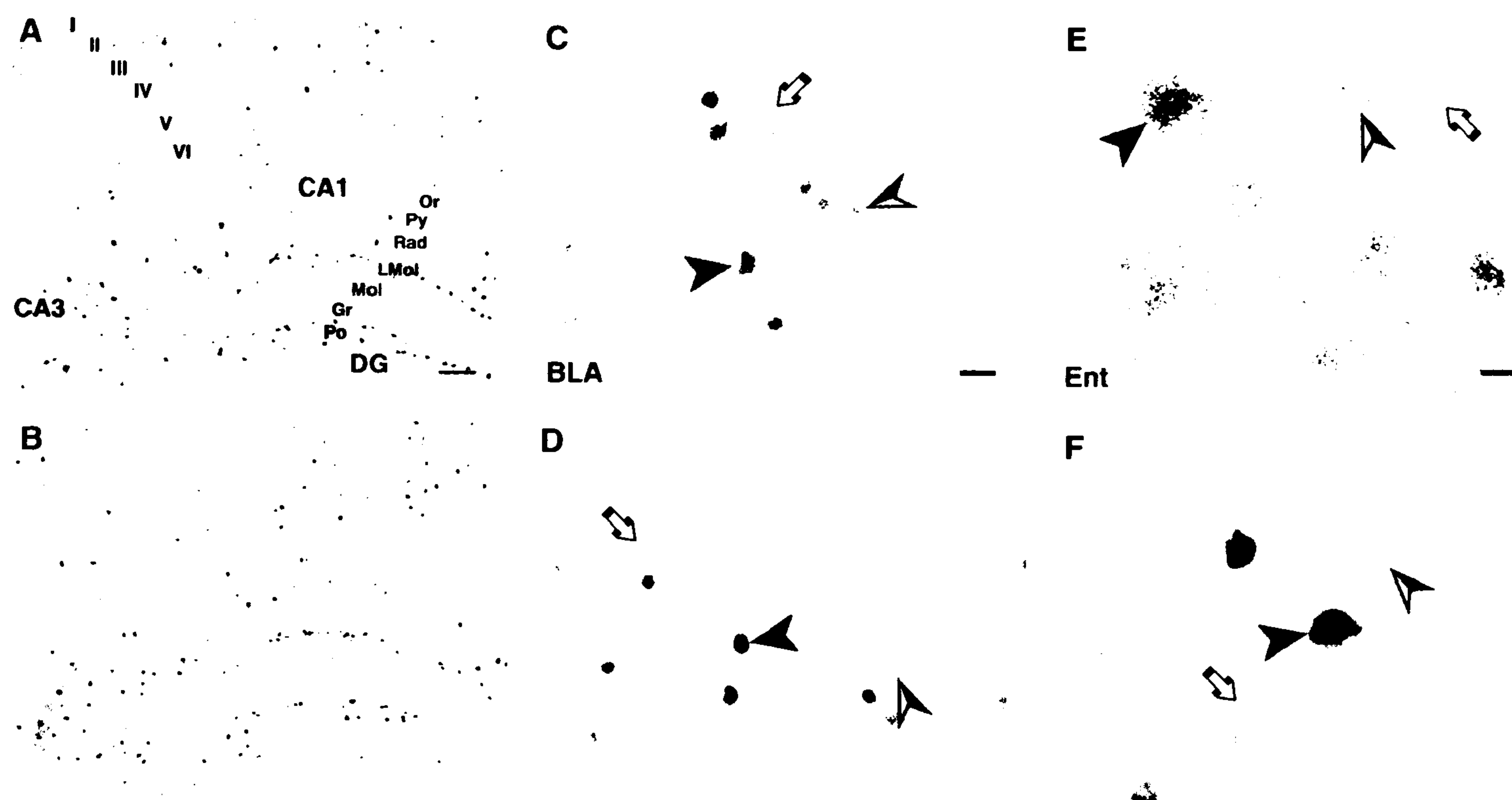


Fig. 2.2. Bright-field micrographs of coronal sections showing CB1 distribution and levels of expression in cortical and limbic structures. Parallel coronal sections from three different brain regions were hybridized either with a ^{35}S -radiolabeled (A,C,E) or with a FITC-labeled (B,D,F) riboprobe. (A,B) hippocampus and dorsal neocortex; (C,D) basolateral amygdaloid nucleus; (E,F) entorhinal cortex. Note that number of labeled cells and intensity of signals are the same when comparing the two ISH techniques. Both high and low CB1-expressing neurons can clearly be classified. The light grey area between CA1 and cortex in (B) is due to nonspecific staining of white matter. Filled arrowhead, high CB1-expressing cell; open arrowhead, low CB1-expressing cell; open arrow, CB1-negative cell. Scale bars: A and B, 150 μm ; C and D, 40 μm ; E and F, 10 μm .

For a detailed analysis of coexpression of CB1 with five neuronal markers, five cortical (hippocampus, neocortex, anterior olfactory nucleus, entorhinal cortex area and amygdaloid area) and two non-cortical areas (dorsolateral caudate putamen and ventromedial hypothalamic nucleus) were chosen, because of their high expression levels and the proposed functional relevance of these regions in cannabinoid physiology and pharmacology (Breivogel & Childers, 1998).

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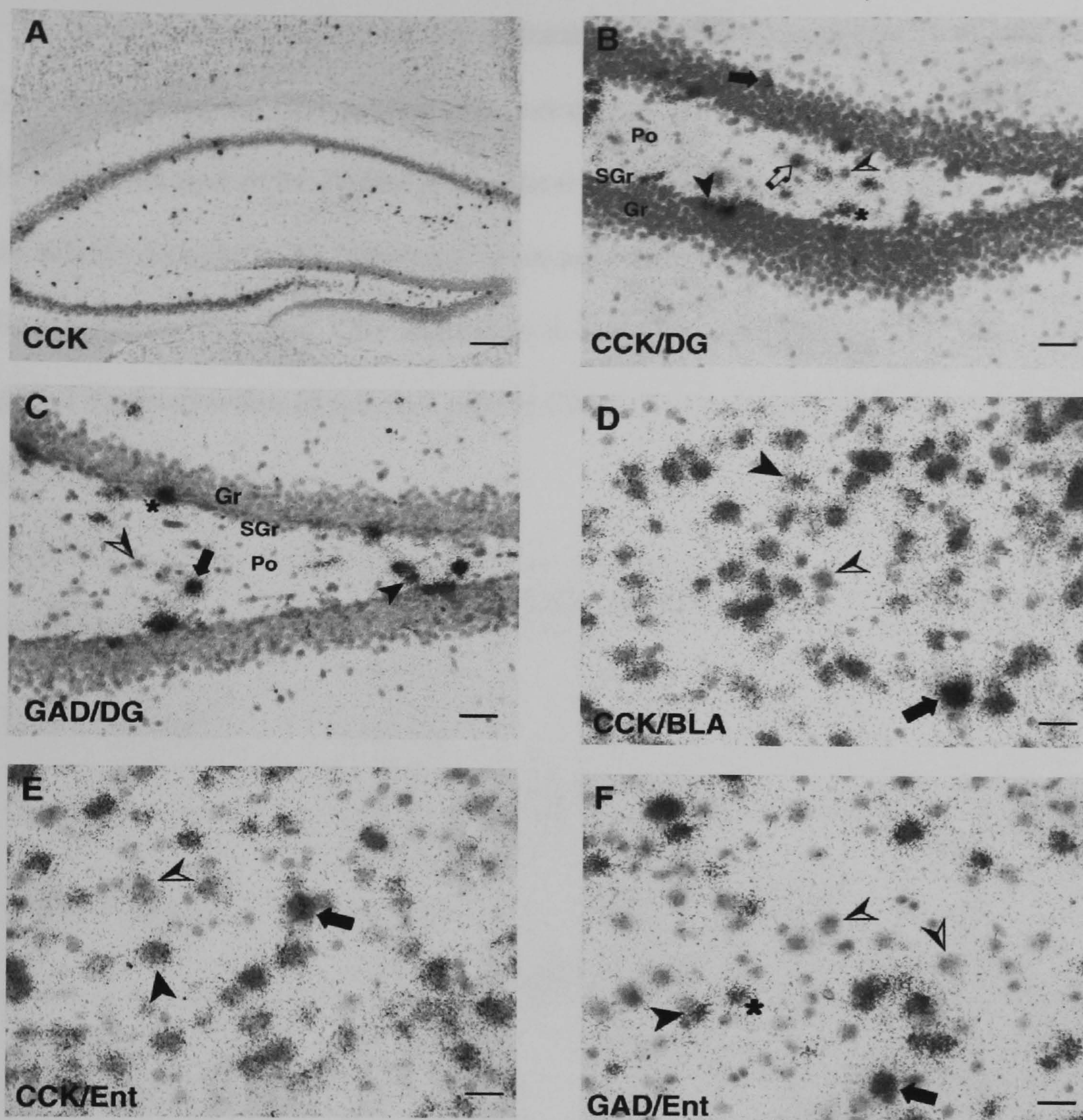


Fig. 2.3. Bright-field micrograph of coronal sections showing examples of coexpression of CB1 (red staining) with CCK and GAD 65 (silver grains), respectively, as detected by double ISH. All sections were counterstained with toluidine blue. (A) Overview of hippocampus double-stained for CB1 and CCK. Note the expression of CCK in pyramidal cells of CA1/CA3 regions. (B) Higher magnification of dentate gyrus from (A) showing high degree of coexpression of CB1 with CCK (filled arrow, filled arrowhead). (C) Dentate gyrus double-stained for CB1 and GAD 65, showing complete coexpression for high CB1-expressing cells. (D) Double staining for CB1 and CCK in basolateral amygdaloid nucleus. Note that CB1 and CCK display almost complete coexpression. (E) Double staining for CB1 and CCK in entorhinal cortex area, showing high degree of coexpression. (F) Double staining for CB1 and GAD 65 in entorhinal cortex area, showing that low CB1-expressing cells do colocalize partly with GAD 65. This is in contrast to CCK as shown in (E). Filled arrow, high CB1-expressing cell coexpressing CCK or GAD 65; filled arrowhead, low CB1-expressing cell coexpressing CCK or GAD 65; open arrow, high CB1-expressing cell not coexpressing CCK or GAD 65; open arrowhead, low CB1-expressing cells not coexpressing GAD 65 or CCK; asterisks, cells expressing only CCK or GAD 65. Scale bars: A, 150 μm ; B and C, 40 μm ; D, E and F, 20 μm .

Double-positive cells are located in granular and subgranular layers of dentate gyrus. GAD 65-negative, but CB1-positive cells contain only low levels of CB1 and are located in the polymorph layer of the dentate gyrus. These cells are often mistaken for interneurons, but are actually thought to be "misplaced" principal cells (Freund & Buszáky, 1996; Tóth & Freund, 1992). Therefore, CB1 expression in these scattered cells is consistent with the general observation that all principal cells of CA1/CA3 area express CB1 at low levels, but lack GAD 65 expression.

		CB1/GAD	CB1/CCK
CA1 Area*	Low CB1	16.5	37.2
	High CB1	26.9	47.8
	Total	43.4 (1154)	85.0 (460)
CA3 Area*	Low CB1	20.7	37.6
	High CB1	21.3	45.3
	Total	42.0 (1366)	82.9 (683)
Dentate Gyrus	Low CB1	23.2	26.8
	High CB1	14.6	26.1
	Total	37.8 (387)	52.9 (187)

Table 2.2. Percentage of coexpression of CB1 in GAD 65- and CCK-positive cells in adult mouse forebrain. Percentages indicate the total fraction of CB1-expressing cells among the GAD 65- and CCK-positive populations. Numbers of analysed cells are in parentheses. Low and high, respectively, indicate the percentages of cells expressing CB1 at low or high levels among the total number of GAD- and CCK-expressing cells. Total means the percentage of all CB1-expressing cells among the total number of GAD- and CCK-positive cells.

* Principal cells in CA1/CA3 areas express low levels of CB1 mRNA and are not included in coexpression data. GAD 65 is not expressed in these cells, but CCK is expressed.

Table 2.2 shows the percentage of CB1 expression among the GABAergic cell population. In CA1/CA3 area, 42-43% of GAD-65 expressing cells also contain mRNA coding for CB1, while the value decreases to 37.8% in the dentate gyrus.

In summary, these results show that, with exception of principal cells in CA1/CA3 and

of cells in polymorph layer, all CB1-expressing cells in the hippocampus are GABAergic interneurons and that CB1-positive cells represent about 40% of GABAergic neurons. In all pyramidal cells of the CA1/CA3 region, CB1 and CCK (Fig. 2.2A,B; Fig. 2.3A) show full coexpression. In hippocampal cells outside the pyramidal cell layer, CCK mRNA is also highly coexpressed with CB1, as shown in Fig. 2.3A,B and Table 2.1. Low CB1-expressing cells show about 70% colocalization in CA1/CA3 and 47% in dentate gyrus. In high CB1-expressing cells, values increase to 91% and 94% in CA1 and CA3, respectively, and to 73% in dentate gyrus. Table 2.2 shows that the fraction of CB1 expressing cells among all CCK-containing neurons is also very high: 85% and 83% and more than 50% in CA1, CA3 and dentate gyrus, respectively. CCK-containing interneurons in the hippocampus and dentate gyrus have been described as a specific subset of basket cells (Gulyás et al., 1991). According to this study, a large fraction of CB1-expressing cells belongs to this particular type of hippocampal interneurons. In addition, considering that pyramidal cells in CA1/CA3 do express both CB1 and CCK (Fig. 2.3A), a very high fraction of all CCK-positive cells in the hippocampus do also express CB1.

Colocalization experiments with calcium binding proteins resulted in a rather different situation. In the hippocampus, PV and CRT are coexpressed with CB1 only at a very low percentage (Table 2.3; Fig. 2.4C). Together with the results obtained with CCK, these observations showed that hippocampal CB1-positive cells can be classified as a subset of basket cells being CCK-positive and PV-negative (Gulyás et al., 1991). Since another subtype of hippocampal interneurons, the axo-axonic cells, were described to contain PV, it is clear from this study that CB1-positive cells belong only to a negligible extent to this interneuronal subtype.

	Low CB1 expressing cells			High CB1 expressing cells		
	C28	PV	CRT	C28	PV	CRT
CA1 Area*	32.4 (294)	1.3 (307)	1.1 (251)	29.1 (204)	1.3 (198)	0.0 (242)
CA3 Area*	29.1 (341)	1.8 (383)	5.2 (313)	22.3 (221)	1.3 (247)	2.5 (247)
Dentate Gyrus	31.5 (144)	0.0 (119)	2.0 (145)	31.5 (144)	2.6 (91)	2.3 (96)
Layers II-III Neocortex	24.9 (983)	0.5 (637)	1.0 (577)	32.8 (471)	1.0 (302)	0.0 (332)
Layers V-VI Neocortex	13.7 (1011)	1.1 (575)	1.3 (525)	13.7 (354)	1.5 (384)	0.0 (414)
Anterior Olfactory Nucleus	ND	4.1 (236)	19.2 (241)	ND	0.0 (91)	0.0 (103)
Entorhinal Cortex Area	16.8 (722)	3.5 (691)	2.8 (612)	39.7 (92)	1.2 (87)	1.3 (75)
Amygdaloid Area	13.1 (787)	4.6 (681)	***	73.5 (89)	1.7 (119)	***
Striatum**	~75	~15	0.0			
Hypothalamus**	~100	0.0	~ 100			

Table 2.3. Percentage of coexpression of calcium binding proteins with CB1 in adult mouse forebrain. Percentages indicate cells expressing low levels or high levels of CB1 that were also labeled with riboprobes specific for calbindin 28k (C28), parvalbumin (PV), or calretinin (CRT). Numbers of analysed cells are in parentheses. Data were collected from 35 (CB1+PV, CB1+CRT) and 30 (CB1+C28) sections from two different brains. ND: not determined.

* Principal cells in CA1/CA3 areas express low levels of CB1 mRNA and are not included in coexpression data. C28 is expressed in CA1 principal cells, PV and CRT are not expressed.

** Due to the uniform distribution of low CB1-expressing cells in the dorsolateral part of striatum and in the ventromedial and anterior hypothalamic nuclei, numbers in these areas reflect an estimation only.

*** CB1/CRT coexpression varies in different amygdaloid nuclei: Basolateral posterior part: 31.7 (184) (low CB1), 14.3 (42) (high CB1); Basolateral anterior part: 8.8 (274) (low CB1), 2.5 (40) (high CB1); Basomedial anterior part: 4.5 (220) (low CB1), 0.0 (37) (high CB1).

Instead, C28 showed a significant percentage of colocalization, in the range of 22% to 32% (Table 2.3; Fig. 2.4A,B). C28-expressing cells in the hippocampus were described as interneurons innervating dendrites of pyramidal cells (Gulyás & Freund, 1996), but they are not considered as basket cells, as these cells innervate mostly the soma of principal neurons (Soriano et al., 1990).

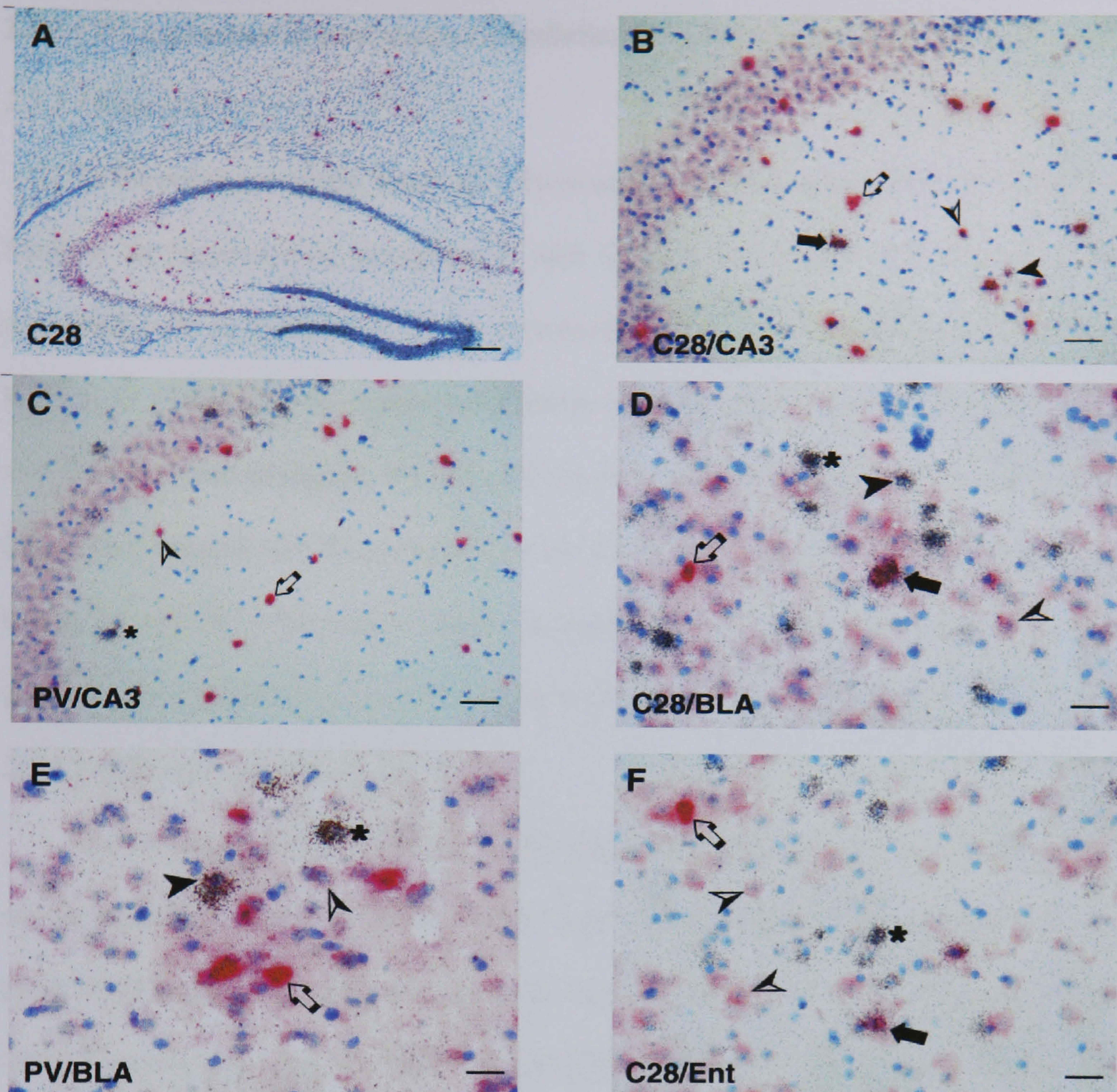


Fig. 2.4. Bright-field micrograph of coronal forebrain sections showing examples of coexpression of CB1 (red staining) with C28 and PV (silver grains), respectively. All sections were counterstained with toluidine blue. (A) Overview of hippocampus double-stained for CB1 and C28. Note the expression of C28 in pyramidal cells of CA1 region and in granular cells of dentate gyrus. (B) Higher magnification of CA3 from (A) showing some coexpression of CB1 and C28 in CA3 area (filled arrow, filled arrowhead). (C) Lack of coexpression of CB1 with PV in CA3 area (open arrow, open arrowhead, asterisks). (D) In basolateral amygdaloid nucleus, C28 is present in many high CB1-expressing cells (filled arrow), but only in a few low CB1-expressing cells (filled arrowhead). (E) Double staining for CB1 and PV in basolateral amygdaloid nucleus, showing only a small fraction of colocalization (filled arrowhead). (F) Double staining of CB1 and C28 in entorhinal cortex area, showing some colocalization (filled arrow). Filled arrows, high CB1-expressing cells coexpressing C28; filled arrowhead, low CB1-expressing cells coexpressing C28 or PV; open arrows, high CB1-expressing cells not coexpressing C28 or PV; open arrowheads, low CB1-expressing cells not coexpressing C28 or PV; asterisks, cells expressing only C28 or PV. Scale bars: A, 150 μm ; B and C, 40 μm ; D, E and F, 20 μm .

2.3.3 CB1 expression in neuronal subpopulations in other cortical regions

2.3.3.1 Neocortex

In the entire neocortex, nearly all CB1-positive cells also express GAD 65 (Table 2.1). Similarly to hippocampus, coexpression with CCK is more than 99% for high CB1-expressing cells in layers V-VI, but is decreased to 77% for low CB1-expressing cells in layers II-III (Table 2.1). No apparent differences in degree of coexpression were observed in various neocortical subregions. PV and CRT showed very low coexpression with CB1 (Table 2.3). Thus, neocortical CB1-positive cells are GABAergic neurons, expressing CCK, but neither PV nor CRT. However, C28 showed some extent of coexpression with CB1, which is higher in layers II-III than in layers IV-V (Table 2.3).

2.3.3.2 Entorhinal/perirhinal cortex area

Entorhinal and perirhinal cortex are the major cortical site of efferents and afferents to and from the hippocampus (Suzuki, 1996; Lopes da Silva et al., 1990). Layers II-III and layers V-VI display very high numbers of low CB1-expressing cells and many scattered high CB1-expressing cells (Fig. 2.1C,D; Fig. 2.2E,F), furthermore pointing out the modulatory functions of cannabinoids in memory processes. Fig. 2.3F and Table 2.1 show that GAD 65 is expressed in nearly all high CB1-expressing cells. Much lower colocalization is observed in the population of cells expressing low levels of CB1. The degree of coexpression with CB1 is higher for CCK than for GAD 65 (Table 2.1, Fig. 2.3E). Taken together, these results indicate that nearly all high CB1-expressing cells are GABAergic neurons containing CCK, while the cells containing low levels of CB1 mRNA are only partly GABAergic, but do express CCK. This is a striking difference to neocortex, where all CB1-positive cells are GABAergic, regardless of the levels of CB1. As observed in hippocampus and neocortex, coexpression of CB1 with CRT and PV is very low (Table 2.3), but coexpression with C28 is 16.8% for low and 39.7% for high CB1-expressing cells (Table 2.3; Fig. 2.4F).

2.3.3.3 *Olfactory system*

Cells expressing low levels of CB1 were detected in piriform cortex and in dorsal regions of the olfactory tubercle, and few high CB1-expressing cells were present in the nucleus of the lateral olfactory tract. The most prominent expression area is located in the anterior olfactory nucleus (Fig. 2.1A), where a high number of low CB1-expressing cells is present interspersed by a low number of strongly positive neurons. All CB1-positive cells are equally distributed throughout all parts of the anterior olfactory nucleus, where the degree of coexpression with GAD 65 and CCK is comparable to that observed in the entorhinal/perirhinal cortex area (Table 2.1). High CB1-expressing cells were found to be positive for both markers in almost 100% of the neurons evaluated, while the degree for low CB1-expressing cells decreases to 35.2% and 89.5% for GAD 65 and CCK, respectively. Thus, as in other cortical regions, high CB1-expressing cells seem to belong to a population of GABAergic neurons containing CCK, while the cells containing low levels of CB1 mRNA partly belong to a CCK/GAD 65-double positive population and partly to another population that is CCK-positive, but GAD 65-negative. For PV and CRT, there is nearly a lack of coexpression with CB1 (Table 2.3). Only few CRT-positive cells (19.2%) were observed in the low CB1-expressing cell population. Together with some nuclei of amygdaloid region (see below), this is the only cortical area in which a significant coexpression of CB1 with CRT was observed.

2.3.3.4 *Amygdaloid region*

CB1 is expressed in several nuclei of the amygdala. Only low levels were detected in the bed nucleus of stria terminalis and in the central amygdaloid nucleus (data not shown). High numbers of both low and high CB1-expressing cells were found, however, in the anterior and posterior parts of the basolateral amygdaloid nuclei and in the anterior part of the basomedial nucleus (Fig. 2.1D; Fig. 2.2C,D). In these latter nuclei, high CB1-expressing cells also contain GAD 65 (Table 2.1). Instead, low CB1-expressing cells have different levels of

GAD 65 coexpression, depending on the nuclei. A gradual decrease along the posterolateral to anteromedial direction was apparent (Table 2.1, see note). In the posterior part of the basolateral nucleus, the percentage of colocalization is 42.7%, while it decreases to 15.0% in the basomedial anterior part. Taken together, almost all high CB1-expressing cells are GABAergic, while a fraction ranging from 57% to 85% for low CB1-expressing neurons is non-GABAergic. CCK highly colocalizes with CB1 in an uniform manner in the amygdaloid nuclei examined (Fig. 2.3D; Table 2.1). Approximately 90% of low CB1-expressing cells and nearly all high CB1-expressing cells contain CCK mRNA. As other cortical areas, amygdala contains low CB1-expressing cells that are CCK-positive, but GAD 65-negative. A distinct population comprising all high CB1-expressing cells and a fraction of low CB1-expressing cells express both GAD 65 and CCK. As Table 2.3 and Fig. 2.4E show, PV is present in very few CB1-positive cells. Similarly to GAD 65, coexpression of CRT also decreases along the posterolateral to anteromedial direction (Table 2.3, see note). C28 displays uniform coexpression throughout the amygdaloid nuclei analyzed. A considerable percentage is observed for low CB1-expressing cells (13.1%), but is increased to 73.5% in high CB1-expressing cells. Thus, in amygdaloid region, about 70-75% of high CB1-expressing cells belong to a population of GABAergic neurons that contains both CCK and C28.

2.3.4 CB1 expression in neuronal subpopulations in non-cortical regions

2.3.4.1 Striatum

CB1 hybridization signals were detected at low levels in many cells throughout the striatum. Nucleus accumbens, ventromedial caudate putamen, globus pallidus and entopeduncular nucleus contain cells expressing quite low levels of CB1. In contrast, dorsolateral caudate putamen showed an intense staining due to a very compact and uniform, but low level expression (Fig. 2.1B,C). Nearly all medium-sized cells in dorsolateral caudate putamen are CB1-positive. Number of labeled cells and intensity of signal decrease along the medioventral axis. Due to uniform distribution of low CB1-expressing cells in this area, it was

not feasible to count coexpressing cells at a single cell resolution. Thus, the numbers reflect an estimation only. Table 2.1 shows the values of coexpression of CB1 with GAD 65 and CCK. Basically all CB1-positive cells in the dorsolateral caudate putamen contain GAD 65 mRNA, while none are CCK-positive. Spiny neurons in caudate putamen express GAD 65, and they constitute approximately 95% of the cells in this region (Ottersen & Storm-Mathisen, 1984). As shown in Table 2.3, many CB1-positive cells (circa 75%) coexpress C28. Scattered cells (ca. 15%) coexpress PV, while there is a lack of colocalization of CB1 with CRT. These results revealed that CB1-expressing cells in dorsolateral caudate putamen are GABAergic neurons that do not express CCK. They might belong to two distinct or partly overlapping populations, which express C28 and PV, respectively.

2.3.4.2 Hypothalamus

Several hypothalamic nuclei display low levels of CB1 mRNA. Strongest hybridization signals were detected in ventromedial (Fig. 2.1D) and anterior hypothalamic nuclei (not shown), where CB1-positive cells are uniformly distributed. There is a lack of coexpression of CB1 and GAD 65 in these hypothalamic nuclei (Table 2.1), as GAD 65 is expressed mainly in hypothalamic areas surrounding the ventromedial and anterior hypothalamic nuclei. Remarkably, there is also a lack of coexpression with CCK (Table 2.1, see note). Among all areas analyzed, this is the only forebrain region in which neither GAD 65 nor CCK colocalize with CB1. C28 and CRT are highly enriched in cell bodies of ventromedial and anteromedial hypothalamus. Thus, they coexpress with CB1 to a high degree (Table 2.3). PV is not expressed in the hypothalamus (Celio, 1990).

2.4 Discussion

The aim of this study was to define in detail the distribution pattern and the neurochemical characteristics of CB1-expressing neurons in the adult mouse forebrain. Therefore, single ISH experiments using ³⁵S-labeled or FITC-labeled CB1 riboprobes, and double ISH experiments using a FITC-labeled CB1 riboprobe in combination with ³⁵S-labeled riboprobes to GAD 65, CCK, PV, C28 and CRT, were performed. CB1-expressing cells can clearly be discriminated into two different populations: one expressing high levels and the other expressing low levels. High CB1-expressing cells are in the great majority GABAergic, characterized mostly by the expression of CCK, the lack of PV and CRT, and, rarely, the presence of C28. Low CB1-expressing cells belong only partly to the same subpopulations of GABAergic cells as high CB1-expressing cells. Remarkably, for low CB1-expressing cells, the presence of CCK characterizes also non-GABAergic neurons. In cortical areas, they can be considered as principal projecting cells. These results are in agreement with recent similar observations obtained by other groups which used single and double immunohistochemistry on rat hippocampus (Tsou et al., 1999; Katona et al., 1999).

The discrimination between high and low CB1-expressing cells is also in agreement with immunohistochemical observations in rats (Tsou et al., 1998a), indicating that the different levels of expression of mRNA are probably connected to different levels of protein expression. The different neurochemical properties of CB1-expressing cells in various forebrain areas are likely to underlie different functional properties.

2.4.1 CB1 and the GABAergic system

Previous immunohistochemical and ISH studies performed in rats (Tsou et al., 1998a; Matsuda et al., 1993) suggested that in cortical structures CB1 receptor is present mainly in GABAergic neurons. Our results indicate that this is true in the mouse forebrain only for high CB1-expressing cells, where GAD 65 colocalizes with CB1 at a degree close to 100%. However, low CB1-expressing cells appear to be less correlated with the GABAergic system.

Pyramidal cells of CA1/CA3 areas in hippocampus express low levels of CB1, but do not express GAD 65. The same is true for many cells in the polymorph layer of dentate gyrus that probably represent misplaced pyramidal cells. From immunohistochemical studies in rats, it is not clear whether CB1 protein is expressed in pyramidal cells of hippocampus. Some authors (Tsou et al., 1998a; Katona et al., 1999; Tsou et al., 1999) report negligible levels of expression of CB1 protein in pyramidal cells of CA1/CA3 region. In contrast, using a different antiserum and different fixation procedures, Pettit et al. (1998) observed a clear expression of CB1 in these cells. This discrepancy might be explained by the fact that different fixation procedures might mask different epitopes or can destroy particular subcellular organelles in which the protein is localized. However, in agreement with results obtained in rats (Matsuda et al., 1993), the present ISH data in mouse clearly revealed that a low but significant amount of CB1 mRNA is expressed in pyramidal cells of CA1/CA3 hippocampal regions. Similarly to hippocampus, in the anterior olfactory nucleus, entorhinal/perirhinal cortex and amygdaloid nuclei, the majority of low CB1-expressing cells is not GABAergic. Only in neocortex, CB1 seems to be completely correlated with the GABAergic system, regardless of the levels of CB1 expression.

Hippocampus, amygdala and entorhinal/perirhinal cortex comprise the so-called limbic system, which is considered to be a central circuit for important brain functions, such as learning and memory, as well as cognition (Miller et al., 1998; Suzuki, 1996). The effects of cannabinoids on memory processes are generally believed to be due to the interaction of CB1 with the GABAergic system (Terranova et al., 1995). Recently, Paton et al. (1998) analyzed the involvement of GABA transmission in the mechanism by which cannabinoids are able to inhibit long-term potentiation (LTP), an electrophysiological correlate of learning and memory in hippocampal slices. Unexpectedly, the authors observed a decrease of GABAergic transmission in the presence of CB1 agonists. Therefore, they concluded that increased GABA activity is not directly involved in the inhibition of LTP by cannabinoids. Moreover,

they speculate that presynaptic CB1 receptors located specifically on terminals of principal glutamatergic neurons, which synapse onto inhibitory interneurons, may mediate the reduction of the excitatory drive of these cells. This is in agreement with the present observation that pyramidal cells of CA1/CA3 express low levels of CB1, but lack expression of GAD 65.

2.4.2 CB1 expression in distinct neuronal subpopulations

In hippocampus, neurochemical characterization of GABAergic neurons defines at least four distinct subpopulations (Freund & Buszáki, 1996). (1) PV-positive GABAergic interneurons are considered as basket cells that innervate perisoma of pyramidal cells and as axo-axonic cells, innervating the proximal part of axons of pyramidal cells (Soriano et al., 1990). (2) CCK-positive GABAergic interneurons represent a different population of basket cells that does not overlap with PV-positive GABAergic neurons, but also innervates the soma of pyramidal cells (Gulyás et al., 1991). (3) C28 is present in cells innervating mid-proximal dendrites of pyramidal cells (Gulyás & Freund, 1996). (4) CRT is characteristic for interneurons specialized in innervating other interneurons (Gulyás et al., 1996). In this study, it was found a high degree of coexpression of CB1 with CCK, some coexpression with C28 and nearly a lack of coexpression with PV and CRT, indicating that CB1-expressing cells are mostly basket cells of the CCK-positive and PV-negative type and, to a lower extent, C28-positive interneurons innervating mid-proximal dendrites of pyramidal cells. As almost no coexpression of CB1 was observed with CRT and PV, CB1 is not present neither in axo-axonic cells, innervating the proximal part of the axons of pyramidal cells nor in interneurons specialized in innervating other interneurons. Interestingly, for low CB1-expressing cells in CA1/CA3 regions of hippocampus, the percentages of coexpression with C28 and with CCK, respectively, appear to be complementary (30% versus 70%). Thus, low CB1-expressing cells that are GABAergic might belong to two distinct interneuronal subpopulations: basket cells (CCK coexpression) and, to a lower extent, cells innervating mid-proximal dendrites

(C28 coexpression). For high CB1-expressing cells there must be an overlap of at least 10-20%, as CB1/C28 coexpression is approx. 20%, whereas CB1/CCK coexpression is more than 90%.

In the limbic system (e.g. entorhinal/perirhinal cortex, amygdala and hippocampus), a significant percentage of low CB1-expressing cells are not GABAergic, but express CCK. In neocortex, many CCK-positive pyramidal cells were described as corticostriatal and corticothalamic projecting neurons (Ingram et al., 1989; Burgunder & Young, 1988; Morino et al., 1994; Senatorov et al., 1997). Many CCK-positive cells also appear to be projecting neurons in entorhinal/perirhinal cortex and amygdala, as, for example, CCK was shown to be present in fibers connecting entorhinal cortex and hippocampus, and in fibres crossing the amygdalo-hippocampal border (Greenwood et al., 1981; Roberts et al., 1984; Fredens et al., 1984). Considering the high levels of coexpression between CB1 and CCK in GAD-negative cells, these observations might indicate an involvement of CB1 in the limbic system that is not only due to an influence on GABAergic inhibitory control, but also to a direct effect on CCK-positive projecting neurons (see below and Fig 2.5). It is interesting to note that neurons expressing CCK and low levels of CB1, but no GAD 65, were not observed in neocortex, where nearly all CB1-expressing cells are GABAergic and, thus, are considered as interneurons.

In caudate putamen, CB1 is coexpressed with GAD 65 and, partly, with C28 and PV, but not with CCK. CB1 was shown to be important for the expression of GAD and neuropeptides such as substance P, dynorphin and enkephalin (Steiner et al., 1999). This is an indication that the relation between neuropeptides and cannabinoids is very important in physiology and pharmacology of the cannabinoid system and that different neuropeptides are involved in different brain areas.

2.4.3 CB1 and CCK

A striking finding of this study is the very high coexpression of CB1 with CCK in cortical areas. Excluding the dentate gyrus, in which coexpression is slightly lower, in all cortical areas analyzed, mRNA encoding the neuropeptide is present in approx. 70-90% of the low CB1-expressing cells and 90-100% of the high CB1-expressing cells. CCK, originally characterized as a gastrointestinal hormone, is one of the most abundant neuropeptides in the brain. The active forms, derived from the precursor pre-procholecystokinin range from 4 to 58 amino acids, but the most abundant peptide is the sulfated octapeptide CCK-8S (Fink et al., 1998). They exert their actions through two receptors, CCK-A, present mostly in the gastrointestinal tract and CCK-B, expressed predominantly in the brain. CCK is involved in feeding, learning and memory, behavioural expression of anxiety, mediation of painful stimuli and is also involved in functions controlled by the dopaminergic, serotonergic and opioid systems (Crawley & Corwin, 1994). It is interesting to note that many functions, in which CCK is involved, are also modulated by cannabinoids. In some cases, the two systems seem to act in an antagonistic fashion. For example, CCK was shown to be hyperalgesic, and CCK receptor antagonists are used to increase the analgesic effects of opioids (Faris et al., 1983; Kellstein & Mayer, 1991; Duggan, 1992), while the antinociceptive effects of cannabinoids are very well known (for a review, see Martin & Lichtman, 1998). CCK is also considered to be a satiety factor (Reidelberger, 1994), while CB1 agonism was proposed to induce feeding behaviour (Mattes et al., 1994). Also, many effects of cannabinoids seem to be mediated by interaction with the dopaminergic and opioid systems (e.g. Castellano et al., 1997; Giuffrida et al., 1999; Tanda et al., 1997; Ledent et al., 1999), both of which are interacting with CCK as well (Crawley, 1991; Duggan, 1992). Given these findings and given the high degree of coexpression of CB1 with CCK, it is tempting to speculate about a possible functional cross-talk between the two systems; e.g., cannabinoids might have an effect on production, processing or release of CCK peptides. Depolarization of cell membrane was shown to

induce release of CCK both *in vitro* and *in vivo* (Raiteri et al., 1993). Compounds that are able to increase intracellular concentration of cAMP enhance depolarization-induced release of CCK, and a decrease of cAMP inhibits CCK release (Beinfeld, 1996). Considering that activation of CB1 can inhibit cAMP production, it appears likely that cannabinoids may modulate the release of CCK. Indeed, preliminary results obtained in our laboratory, indicate a significant dose-dependent inhibitory effect (ca. 50% of maximal reduction) of the potent CB1 agonist WIN55,212-2 (1 μ M and 0.1 μ M) on the KCl-induced release of CCK from mouse cortical slices (Marsicano & Lutz, unpublished observations).

Possible interactions between CB1 and CCK may also involve the regulation of CCK synthesis by cannabinoids. In this regard, it is interesting to note a possible involvement of both the receptor and the peptide in some pathophysiological conditions, such as schizophrenia. Based on similar scores in psychodiagnostic tests between schizophrenic patients and healthy volunteers to which cannabinoids were administered, and based on the interaction of cannabinoids with the dopaminergic and glutamatergic system, a "cannabinoid hypothesis" of schizophrenia has recently been proposed, suggesting an increased pathological cannabinoid tone in schizophrenic patients (Emrich et al., 1997; Schneider et al., 1998; Leweke et al., 1999). On the other hand, abnormally decreased levels of CCK mRNA were observed in schizophrenic subjects, but not in Alzheimer patients in discrete cortical areas, such as entorhinal cortex (Gabriel et al., 1996; Bachus et al., 1997). It would be tempting to relate these observations with our CB1/CCK coexpression study and to propose that an increased stimulation by cannabinoids might lead to decreased levels of CCK in distinct cortical brain areas.

2.4.4 From anatomy to function: putative sites of action of cannabinoids in cortical areas

CB1 is distributed throughout the central nervous system of mammals in greater abundance than most of the other known G-protein-coupled receptors (Herkenham et al.,

1990). Therefore, it would be an error to underestimate the physiological importance of cannabinoids in cells that express CB1 at low levels, because such expression is in any case very high as compared to other identified receptors in the brain. For example, despite the rather low levels of CB1 mRNA expressed by single cells in caudate putamen and in other subcortical areas (e.g. preoptic area in hypothalamus), the CB1 receptor seems to exert essential functions in locomotor activity and in hypothermia, respectively (Steiner et al. 1999; Giuffrida et al., 1999; Zimmer et al., 1999; Breivogel & Childers, 1998).

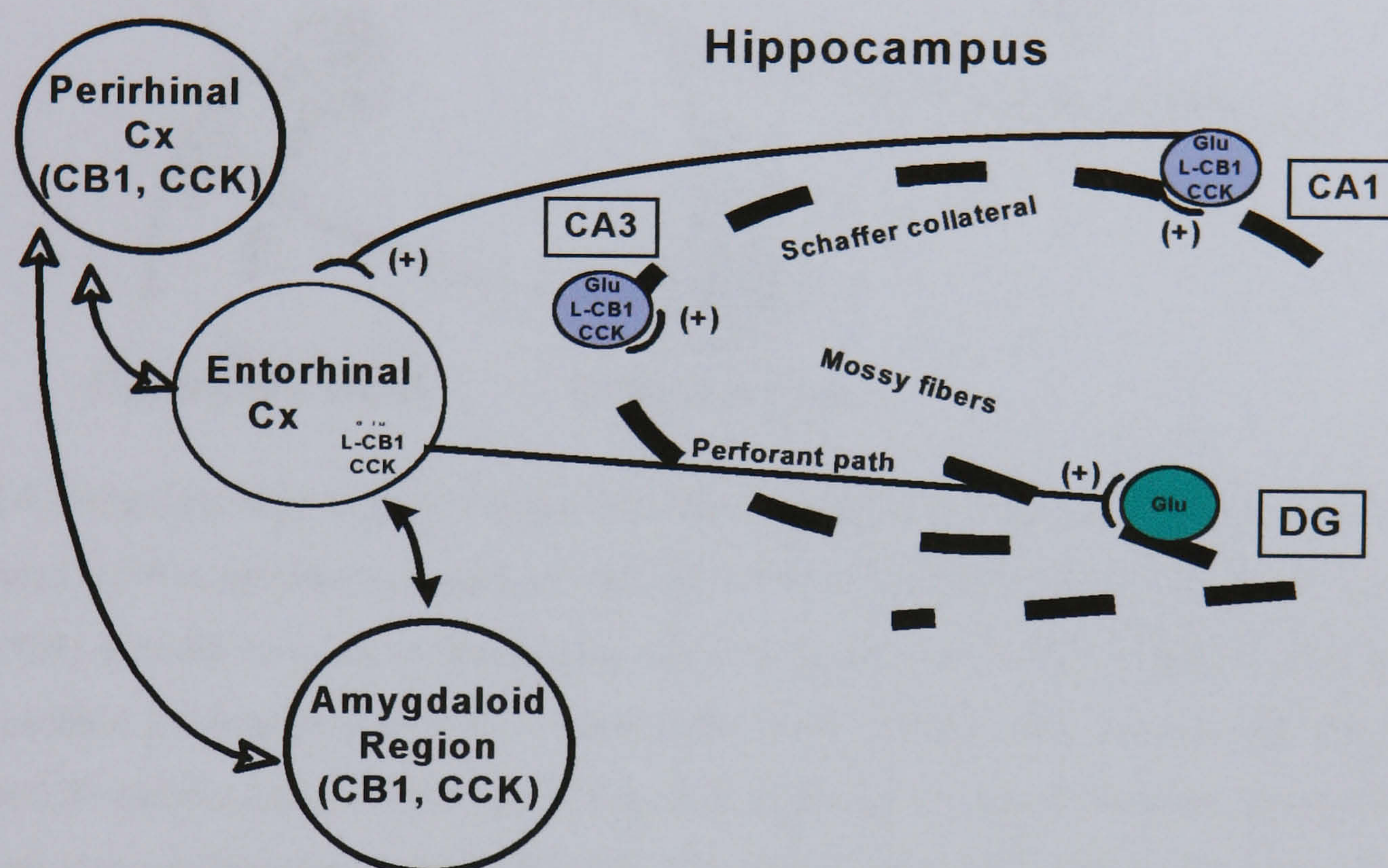


Fig. 2.5. Schematic representation of the connectivity of hippocampus and cortical areas that are proposed to be modulated by the cannabinoid system. CB1 is present at low but significant levels (L-CB1) in principal neurons participating in the "trisynaptic loop" between entorhinal cortex and hippocampus. Due to the high number of low CB1-expressing cells (that are GAD-negative and CCK-positive) in perirhinal and amygdaloid areas, it appears likely that cannabinoid signaling might also modulate neuronal transmission between perirhinal, entorhinal and amygdaloid area. Note the high degree of CB1/CCK coexpression in projecting neurons, suggesting an involvement of the neuropeptide in cannabinoid action.

CB1 is present in many cells belonging to the so-called "trisynaptic loop" (Knowles, 1992) between entorhinal cortex and hippocampus (Fig. 2.5). The present results show that a high percentage of low CB1-expressing cells are GAD 65-negative and CCK-positive and, thus, are likely to be principal projecting neurons. Cannabinoid activity on these receptors,

therefore, might result in a direct modulation of the "trisynaptic loop". Other likely sites of action of cannabinoids in the hippocampus and probably also in other forebrain areas (e.g. neocortex) are the local GABAergic inhibitory circuits (Fig. 2.6).

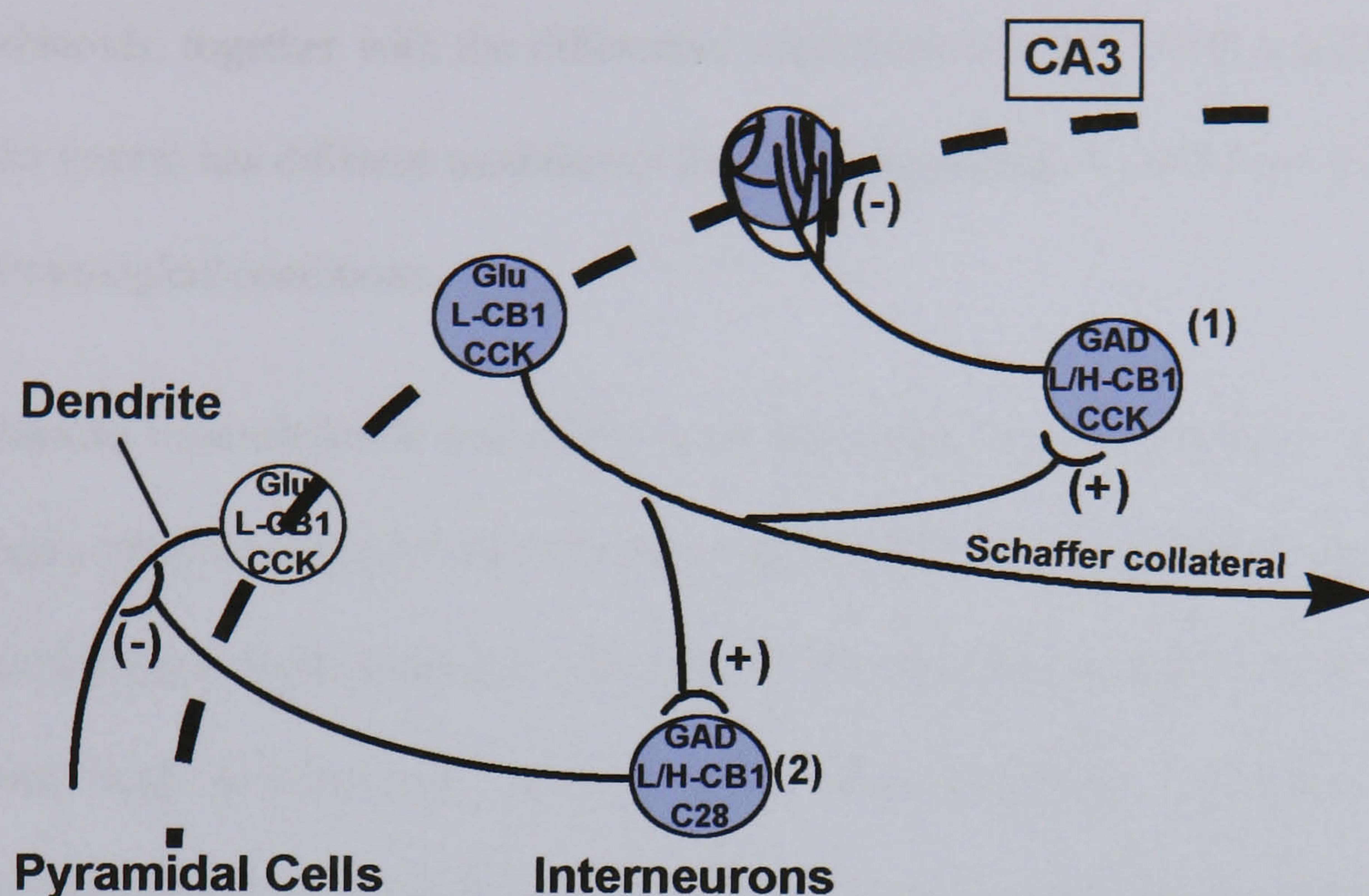


Fig. 2.6. Schematic representation of local hippocampal circuits in CA3 area that are proposed to be modulated by the cannabinoid system. (1) and (2): CB1 is expressed both at low and at high levels (L/H-CB1) in GAD 65-positive interneurons. (1): The majority of neurons (70-80%) belongs to the CCK-positive, PV-negative subfamily of basket cells, innervating the soma of pyramidal cells. (2): 20-30% of CB1-positive cells belong to the C28-positive subfamily of interneurons that innervate the mid-proximal tract of dendrites of pyramidal cells. For clarity, pyramidal cells are represented as three different cells, but a direct feed-back between one pyramidal cell and one interneuron is also possible. (+) and (-) indicate an excitatory and an inhibitory effect of synaptic transmission, respectively. In summary, the cannabinoid system might be involved in local inhibitory GABAergic circuits, by modulation of basket and mid-proximal dendritic inhibitory cells.

In hippocampal slices, cannabinoids were indeed shown to cause a reduction of release of several neurotransmitters, including glutamate and GABA (Shen et al., 1996; Katona et al., 1999), possibly suggesting differential modulatory effects on different cell types.

In summary, these diverse sites of action could indicate a balanced modulatory effect of cannabinoids on principal projecting neurons and on interneurons. Endocannabinoids are believed to be locally produced, released and rapidly inactivated (for reviews, see Di Marzo & Deutsch, 1998; Di Marzo et al., 1998; Piomelli et al., 1998). Anandamide and 2-

arachidonylglycerol, the two major endocannabinoids described so far, were shown to be released upon chemical or electrical stimulation of neurons (Stella et al., 1997; Giuffrida et al., 1999; Piomelli et al. 1998). This locally induced release and the rapid inactivation of endocannabinoids, together with the differential expression of CB1, could suggest that the cannabinoid system has different modulatory functions depending on particular physiological or pathophysiological conditions.

Glutamate transmission is one of the most important events that occur in the brain. The expression study described in this Chapter suggests that the cannabinoid system might be a potential modulator of glutamatergic transmission. It is therefore possible that other systems that interact with glutamatergic neurons have some functional cross-talks with the cannabinoid system. As an example, strong connections are known between the nitric oxide system and glutamate transmission (Bredt & Snyder, 1994; East et al., 1996; Contestabile, 2000). In this view, a putative functional interaction between the cannabinoid system and the nitric oxide pathway was hypothesised and experiments in this direction will be described in the next Chapter, by the use of neuronal nitric oxide synthase-deficient mouse mutants.

Chapter 3

Differential role of the nitric oxide pathway on Δ^9 -THC-induced central nervous system effects in mouse

The work described in this Chapter is in press as Azad, Marsicano, Eberlein, Putzke, Zieglgänsberger, Spanagel and Lutz, European Journal of Neuroscience. Azad and Marsicano share the first authorship. My contributions were the *in situ* hybridization experiments and their quantitative evaluation, whereas Dr. Shahnaz Azad performed the behavioural experiments. Results are given without discriminating the various contributions in order not to disrupt the completeness of the experimental strategy.

3.1 Introduction

Δ^9 -tetrahydrocannabinol (Δ^9 -THC), the major psychoactive constituent of *C. sativa*, is known to exhibit a variety of central effects including hypothermia, antinociception and changes of locomotor activity (Felder & Glass, 1998; Ameri, 1999; Pertwee, 1997). These effects are mainly mediated by the "brain-type" cannabinoid receptor CB1, a seven transmembrane G-protein-coupled receptor predominantly expressed in the central nervous system (Pertwee, 1997). The entity of cannabinoid receptors with binding, synthesis, release and degradation of their endogenous ligands is generally referred to as a novel neuromodulatory system called the cannabinoid system (Di Marzo et al., 1998). Results of recent studies imply that the cannabinoid system is likely to interact with several neurotransmitter systems such as the GABAergic, dopaminergic, opioid and glutamatergic systems (Herkenham et al., 1991; Bidaut-Russell & Howlett, 1991; Glass & Felder, 1997; Pertwee & Wickens, 1991; Manzanares et al., 1999; Ameri, 1999; Piomelli et al., 2000; Hampson et al., 1998b). There is also a general consensus that the nitric oxide (NO) pathway is strongly linked to the glutamatergic

system (Bredt & Snyder, 1994; East et al., 1996; Contestabile, 2000), but also to the GABAergic, dopaminergic and opioid ones (e.g. Jayakumar et al., 1999; Liu, 1996; Herman et al., 1995). NO is an intra- and extracellular messenger, which is produced by the nitric oxide synthase (NOS). There are three NOS genes encoding the respective isoforms: endothelial (eNOS), inducible (iNOS) and neuronal NOS (nNOS) (Garthwaite & Boulton, 1995; Huang & Lo, 1998; Contestabile, 2000) with the latter being the focus of the present investigation. nNOS is a calcium/calmodulin-dependent enzyme which was found first in neurons (Bredt & Snyder, 1994). It is considered to participate in a variety of physiological and pathological processes such as neuronal plasticity and neurotoxicity (Dawson et al., 1998). In addition, NO is known to be involved in the effects of many centrally acting anaesthetic and analgesic drugs (Johns et al., 1992; Tonner et al., 1997; Ferreira et al., 1991).

In vitro studies have shown that there is a link between cannabinoid signalling through cannabinoid receptors and NO pathway in vertebrate brain. CP-55940, a potent agonist of CB1, is able to decrease the release of NO from endotoxin/cytokine-activated rat microglial cells (Waksman et al., 1999). Similarly, several potent CB1 agonists were shown to inhibit KCl-induced activation of NOS from primary cerebellar cultures, whereas the cannabinoids had no effect on basal NOS activity (Hillard et al., 1999). *In vivo* pharmacological studies have analysed the effects of NOS inhibitors on cannabinoid-induced behavioural and pharmacological responses (Thorat & Bhargava, 1994; Spina et al., 1998). Both groups did not observe any effect of NOS inhibitors on analgesic, hypothermic or cataleptic effects of acute cannabinoid administration in mice and rats, respectively. However, only Spina et al. (1998) described an inhibitory effect of the NOS inhibitor N(omega)-nitro-L-arginine methyl ester (L-NAME) on tolerance to hypothermic and cataleptic effects induced in rats by chronic treatment with the potent cannabinoid agonist WIN 55,212-2. On the contrary, Thorat and Bhargava (1994), using

the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA), did not observe any similar effect on tolerance to analgesic and hypothermic effects induced by chronic treatment with Δ^9 -THC. Even though such studies seem to indicate that NOS and NO pathway are not involved in the acute effects of cannabinoid agonists, the discrepant results on tolerance induced by chronic treatment with cannabinoids, could indicate that *in vivo* administered NOS inhibitors may have different efficacies in blocking NOS activity, e.g. depending on the species used in the experiments or on time, route or dosage of administration.

For this reason, a genetic approach was chosen to test the existence of a specific functional link between behavioural and pharmacological actions of cannabinoids and the nNOS/NO pathway. Therefore, body temperature, nociception and locomotion were measured both in neuronal nitric oxide synthase knock-out (nNOS-KO) (Huang et al., 1993) and wild-type (WT) control mice after intraperitoneal application of Δ^9 -THC. In addition, the distribution of CB1 and nNOS transcripts was determined in adult mouse brains using *in situ* hybridization to reveal possible changes in CB1 gene expression in nNOS-KO as compared with WT mice, and to reveal the brain areas, where CB1 and nNOS were coexpressed in the same neuron.

3.2 Materials and methods

3.2.1 Animals

Homozygous males with a deficiency in the neuronal nitric oxide synthase gene (nNOS-KO) and wild-type male control mice (WT) were used for the study. Targeted disruption of the nNOS gene is described in Huang et al. (1993). The genetic background consisted of a combination of the strains 129/Sv and C57BL/6J, with a predominance of C57BL/6J, as mutants were backcrossed for three generations onto C57BL/6J and were then intercrossed to obtain experimental animals. As it is known that male nNOS-KO mice show a markedly increased aggressive behaviour (Nelson et al.,

1995), all animals were housed individually in the same temperature- and humidity-controlled room with a 12 hour light-dark cycle (light from 07:00 to 19:00) and with access to food and water *ad libitum*. At the time of investigation, animals were 9 to 10 weeks old and had a body weight of 21-26 g. All animals were drug naive and were injected only once with Δ^9 -THC or vehicle. All behavioural experiments were performed between 9:00 a.m. and 1:00 p.m. All behavioural and molecular investigations were evaluated in a blinded way. The experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

3.2.2 Drugs and chemicals

Δ^9 -THC was purchased (Sigma, Deisenhofen, Germany) as a 100 mg/ml (w/v) solution in 100% ethanol. Immediately before injection Δ^9 -THC was diluted 1:100 in 45% β -hydroxy-cyclodextrin (RBI/Sigma, Deisenhofen, Germany) and stirred for 10 min at 37°C. As a vehicle control, 45% β -hydroxy-cyclodextrin containing 1% ethanol was used. All drugs were administered i.p. with an injection volume of 10 ml/kg body weight. For behavioural tests, 10 mice of each genotype received Δ^9 -THC or vehicle, respectively. In a pilot study carried out in C57BL/6 mice using various doses of Δ^9 -THC, a dose of 10 mg/kg Δ^9 -THC was found to be the lowest dose leading to clear analgesic and hypothermic effects.

3.2.3 Behavioural testing

To reveal possible differences between the nNOS-KO and WT mice concerning behavioural and physiological reactions to Δ^9 -THC, common cannabinoid-induced effects such as changes in nociception, body temperature and locomotor activity were assessed in both genotypes. Antinociceptive effects were measured 30, 60 and 90 min after injection of Δ^9 -THC or vehicle using a hot-plate analgesia meter (Bachofar, Reutlingen, Germany). The latency until mice showed first signs of discomfort (licking or

flinching of the paws or jumping) on the plate, which was heated to 55 ± 0.5 °C, was recorded. A cut-off-time of 60 sec was set to prevent tissue damage. Δ^9 -THC-induced hypothermia was determined using an infrared thermometer (C-1600, Linear Laboratories, Fremont, California, USA) which was placed between the forepaws at a distance of exactly 3 cm. Body temperature was recorded immediately before as well as 60 and 120 min after injection of drug or vehicle. Spontaneous locomotor activity was assessed by an automated open field system (box size 32 x 32 cm; illumination of 40-60 lux, MOTION, TSE GmbH, Bad Homburg, Germany). 15 min after injection of Δ^9 -THC or vehicle, animals were tested individually for 30 min. The cumulative horizontal distance the animals moved within the box was recorded.

3.2.4 *In situ* hybridization

In situ hybridization on CB1 mRNA was performed to reveal possible differences of CB1 mRNA levels between the two genotypes. Six untreated mice of each genotype of the same age and weight as those tested in behavioural experiments were used. Animals were killed with CO₂, brains were rapidly removed, snap frozen on dry-ice and stored at -80°C. Coronal sections of 20 µm were cut on a cryostat microtome (HM 500, Microm, Walldorf, Germany). Sections were mounted onto frozen SuperFrost/Plus slides (Menzel, Braunschweig, Germany), dried and stored at -20°C. For hybridization, slides were warmed up for 45 min at room temperature. Labelling of the riboprobes and *in situ* hybridization with ³⁵S-labelled riboprobes complementary to the mRNA of the mouse CB1 were performed as described by Marsicano and Lutz (1999) and in Chapter 2. The slide-mounted sections were apposed to autoradiographic films (Kodak Biomax MR film, Integra Biosciences GmbH, Fernwald, Germany) for 11 hours. Developed films were illuminated with a light box and sections were scanned as grey scale images with 256 grey values using a computer-assisted video camera. Mean densities of the regions of interest were measured using Object-Image1.62 for Macintosh with a value of zero

reflecting white and 255 reflecting black.

For the colocalization of CB1 and nNOS, double *in situ* hybridization experiments were performed according to Marsicano and Lutz (1999) (see also Chapter 2). nNOS template was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) using the 5' primer 5'-CCT GGT GGA GAT TAA CAT TGC-3' and the 3' primer 5'-CTG GTA CTG CAA CTC CTG ATT-3'. Amplification product was 1197 bp and covered positions 1985-3182 of Genbank accession number NM008712. PCR product was cloned in pBluescript KS⁺ (Stratagene, CA, USA), and the clone was confirmed by sequencing. Sense and antisense riboprobes were obtained as described in Marsicano and Lutz (1999), linearizing the plasmid with the restriction enzyme BamHI and EcoRI, respectively, and using T3 and T7 RNA polymerase to obtain ³⁵S-labelled riboprobes. CB1 and nNOS sense probes gave no detectable signals. Double *in situ* hybridization experiments were performed using CB1 antisense riboprobe labelled non-radioactively with FITC, and using ³⁵S-labelled nNOS probe. The FITC signal was revealed using the Vector Red kit (Vector Laboratories, CA, USA) for 24-36 hours. The radioactive signal was revealed by dipping the slides in photographic emulsion (NTB-2, Kodak) and developing after 10-20 days.

3.2.5 Statistical analysis

Hot plate analgesia and temperature were first analyzed using analysis of variance (ANOVA) with repeated measurement and the genotypes and therapy as between-subject-factors. In case of significant interactions, univariate F-tests were performed in order to evaluate significant differences at the respective time points. Univariate analysis of variance was used for statistical evaluation of the open field observations. Results of *in situ* hybridization of the different brain regions were evaluated using multivariate analysis of variance. In all cases, a p-value < 0.05 was considered as statistically significant. All results are shown as mean and standard error of the mean (SEM) of the

absolute values. As the WT mice showed a lower basal activity in the open field system than the nNOS-KO mice, Δ^9 -THC-induced changes in locomotor activity were evaluated and expressed as a percentage of the mean activity of the vehicle-treated group for each genotype.

3.3 Results

3.3.1 Behavioural and pharmacological assessments

Measurement of body temperature (Fig. 3.1A) revealed no differences in basal values between the genotypes (Wilks multivariate test of significance; influence of genotype: $F(2,34)=0.750$; $p=0.480$). However, in contrast to the analgesic effects, there was a significant interaction between genotype and treatment (Wilks multivariate test of significance; effect of treatment: $F(2,34)=5.87$, $p<0.01$; effect of type x treatment: $F(2,34)=3.407$, $p<0.05$).

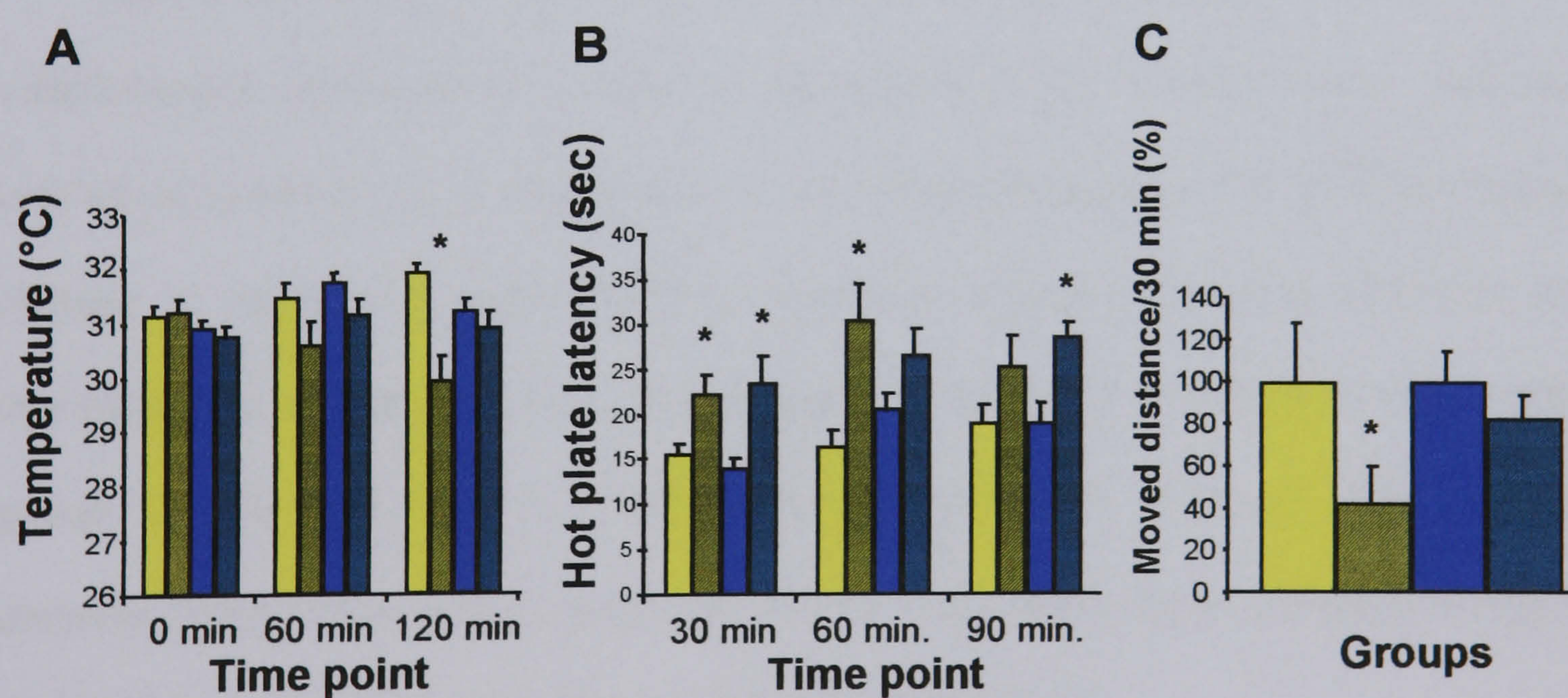


Fig. 3.1. Effects of intraperitoneal application of 10 mg/kg Δ^9 -THC in nNOS knock-out (KO) and wild-type control mice (WT). A. Body temperature B. Hot-plate latencies C. Spontaneous locomotion activity. Yellow bars, WT mice treated with vehicle; hatched yellow bars, WT mice treated with Δ^9 -THC; blue bars, KO mice treated with vehicle; hatched blue bars, KO mice treated with Δ^9 -THC. Data are shown as mean and SEM; asterisks, $p < 0.05$ vs. vehicle.

A hypothermic effect of 10 mg/kg Δ^9 -THC was observed only in the wild-type

mice reaching statistical significance at 120 min after injection (Univariate F-tests, p-value < 0.05).

The effect of Δ^9 -THC on nociceptive responses to acute pain were assessed by using the hot plate test, which is considered to involve mainly supraspinal mechanisms of the nociceptive system. As shown in Fig. 3.1B, hot plate latencies after injection of vehicle did not differ between the mutant mice and the wild-type controls. Acute intraperitoneal application of 10 mg/kg Δ^9 -THC exerted significant antinociceptive effects as measured by an increase of hot plate latencies in both the KO and the WT mice. Significant effects of Δ^9 -THC on latency was observed at all three time points analyzed (Univariate F-tests, p-value < 0.05), but there were no differences in the antinociceptive reactions to Δ^9 -THC between the genotypes. (Wilks multivariate test of significance; effect of treatment: $F(3,34)=9.505$, $p<0.0001$; influence of genotype: $F(3,34)=0.151$, $p=0.928$).

Open-field observations revealed a significantly higher locomotor activity of the vehicle-treated nNOS-deficient mice as compared to the vehicle-treated wild-type controls (Univariate test of significance, $p<0.01$, data not shown). The Δ^9 -THC-induced changes in locomotion were therefore evaluated separately in each genotype and expressed as a percentage of the mean distance moved by the respective vehicle-treated group. The results in Fig. 3.1C show that injection of 10 mg/kg Δ^9 -THC led to a decrease of movement in both genotypes, but this reduction reached significance only in the wild-type animals (Univariate test of significance, $p<0.05$).

3.3.2 Cannabinoid receptor CB1 mRNA expression

As a result of the different effects of Δ^9 -THC in the two genotypes regarding body temperature and locomotor activity, the levels of CB1 mRNA were determined using *in situ* hybridization to reveal possible changes of CB1 gene expression in the nNOS-KO as compared to the WT mice. Evaluation was performed by measuring the mean density

values on the autoradiographic films and included those brain areas which are proposed to be involved in mediating the respective cannabinoid-induced effects (Breivogel & Childers, 1998; Ameri, 1999; Martin & Lichtman, 1998). Periaqueductal grey, dorsal raphe, ventroposterolateral thalamus and amygdala were investigated regarding antinociceptive effects. Median preoptic area and ventromedial hypothalamus were evaluated in respect to hypothermia. Caudate putamen, globus pallidus and substantia nigra were analysed regarding locomotor activity.

Area	Wild-type mice	Knock-out mice
Periaqueductal grey	63.2 \pm 5.5	63.2 \pm 7.4
Dorsal raphe	38.9 \pm 6.5	38.3 \pm 4.7
Ventroposterolateral thalamus	10.2 \pm 3.2	8.4 \pm 2.4
Amygdala	144.1 \pm 6.7	128 \pm 6
Median preoptic area	107 \pm 10.7	101 \pm 17.8
Ventromedial hypothalamus	151.2 \pm 10.3	114.8 \pm 23.9 *
Caudate putamen	201 \pm 8.9	182 \pm 8.7 *
Globus pallidus	21.3 \pm 3.6	18.0 \pm 1.4

Table 3.1. Density values of the regions of interest reflecting CB1 mRNA levels in nNOS knock-out (KO) and wild-type (WT) control mice. Hybridization was made using the same probe and in the same experiment. Slides from KO and WT were exposed to the same autoradiographic film. Data are shown as mean and SEM of averaged values from four sections from each mouse. N=6 mice for each genotype; * $p < 0.05$ between the two genotypes.

Statistical analysis revealed a significant interaction between the genotype and the mean density values reflecting CB1 mRNA levels (Wilks multivariate test of significance; effect of genotype: $F(8,1)=12371.84$, $p<0.01$). A subsequent detailed analysis of the particular areas (Table 3.1) showed that there were significantly lower levels of CB1 mRNA in the ventromedial hypothalamus and the caudate putamen of the KO in comparison to the WT mice ($p<0.05$). No significant differences between the two genotypes were observed in either of the other regions. Representative examples of the

in situ hybridization analysis are depicted in Fig. 3.2, showing decreased CB1 expression in the ventromedial hypothalamus and caudate putamen, as compared with areas where no significant differences were observed (i.e. periaqueductal grey and median preoptic area).

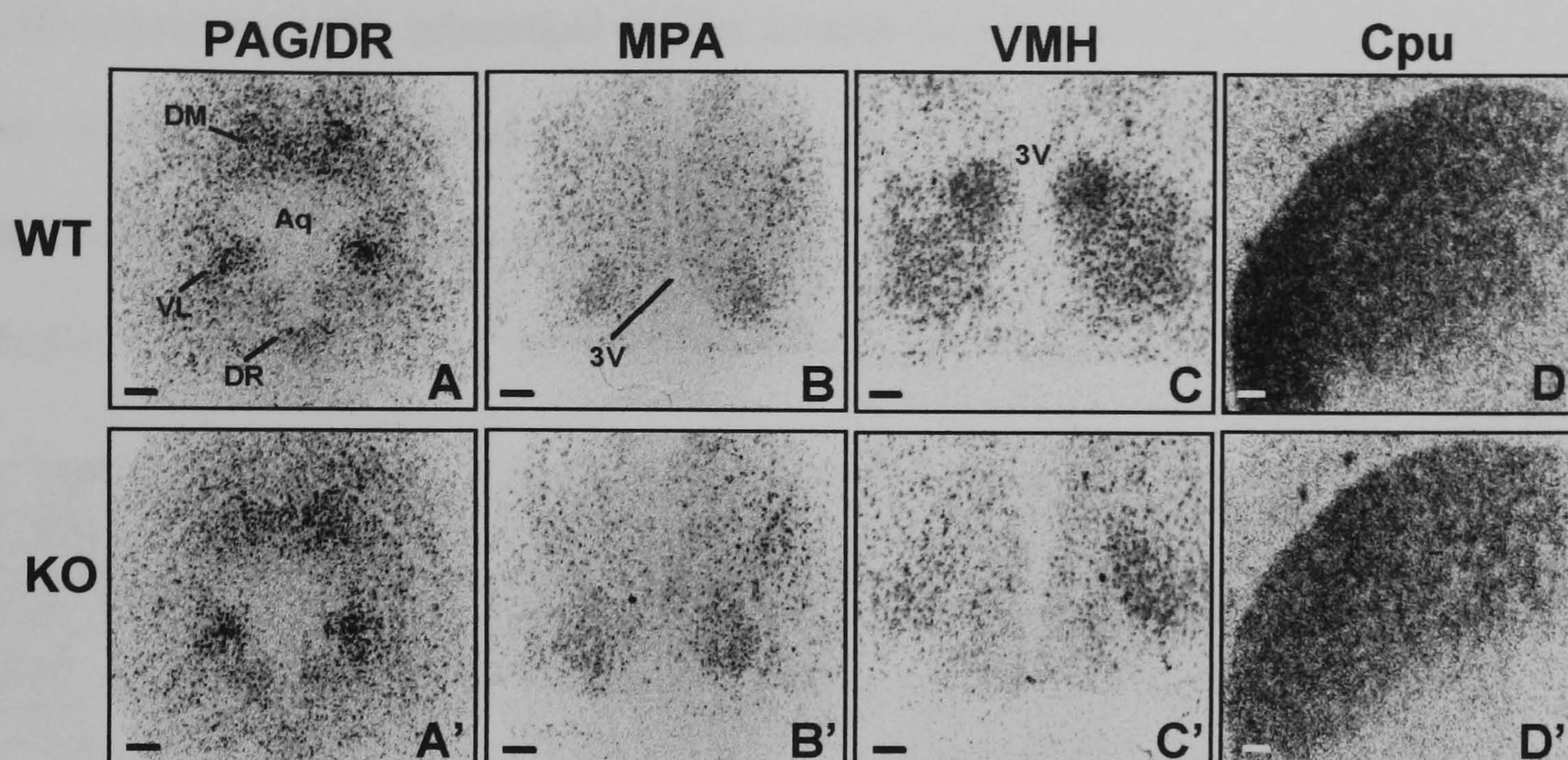


Fig 3.2. Reversed dark-field micrograph of coronal sections showing examples of CB1 mRNA expression levels in different brain areas, as compared between WT and nNOS-KO animals. (A, A') Periaqueductal grey and dorsal raphe. (B, B') Medial preoptic area. (C, C') Ventromedial hypothalamus. (D, D') Dorsolateral caudate putamen. Note the lower expression of CB1 mRNA in VMH and Cpu in the nNOS-KO. Abbreviations: 3V, third ventricle; Aq, aqueductus; Cpu, caudate putamen; DR, dorsal raphe; MPA, medial preoptic area; PAG, periaqueductal grey (DM, dorsomedial; VL, ventrolateral); VMH, ventromedial hypothalamus. Scale bars: 100 μ m.

3.3.3 Analysis of CB1/nNOS coexpression

Since mice lacking nNOS responded to Δ^9 -THC treatment at a reduced extent in particular behavioural paradigms and, moreover, showed a decreased expression of CB1 in the brain areas that are considered to be involved in the behavioural effects of Δ^9 -THC, the question arose whether these results are based on a cell autonomous event, i.e. on changes in the physiology of neurons expressing both nNOS and CB1, or whether the differential responses of nNOS-KO mice to Δ^9 -THC are the consequence of an altered cross-talk between different neurons.

To answer this question, a double *in situ* hybridization study was carried out on

forebrain tissue of wild-type animals to describe the expression of nNOS and CB1 at a single cell level. The mouse forebrain contains cells that express both high and low levels of CB1 mRNA and protein (Matsuda et al., 1993; Tsou et al., 1998a; Pettit et al., 1998; Marsicano & Lutz, 1999, Chapter 2). While cortical areas possess both high and low CB1-expressing cells, subcortical regions contain mostly low CB1-expressing cells that are usually densely packed. Cortical areas, such as the hippocampus (Fig 3.3A), neocortex (Fig. 3.3B), entorhinal cortex or basolateral amygdala (data not shown) display a very low extent of coexpression of CB1 with nNOS.

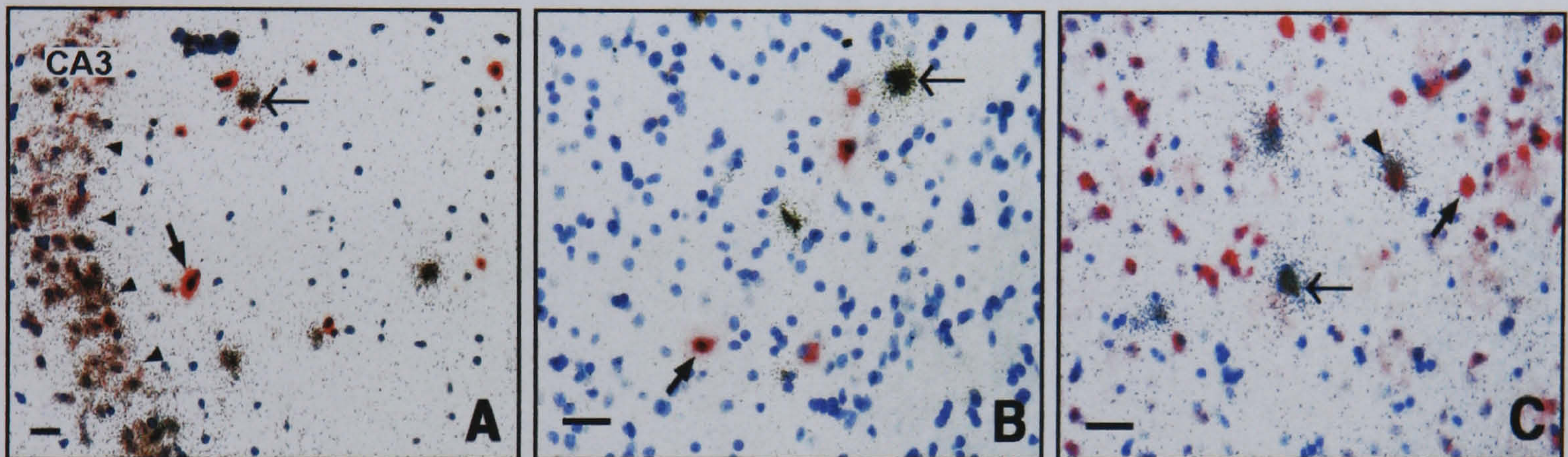


Fig. 3.3. Bright-field micrographs of coronal sections showing examples of coexpression of CB1 (red staining) with nNOS (silver grains), respectively, as detected by double ISH in WT mice. All sections were counterstained with toluidine blue. (A) CA3 area of hippocampus, showing coexpression only in the principal cell layer. (B) Double staining for CB1 and nNOS in neocortex. Almost no coexpression is observed. (C) Dorsal caudate putamen. Note the scattered distribution of nNOS-positive cells and the diffuse staining of CB1. A certain fraction of nNOS-expressing cells contain also CB1 mRNA. Filled arrows: CB1-expressing cell. Open arrows: nNOS-expressing cell. Arrowheads: cells coexpressing CB1 and nNOS transcripts. Scale bars: 20 μ m.

Only principal cells in CA1 and CA3 regions of the hippocampus (Fig. 3.3A) and a few cells in the basolateral amygdala (data not shown) express low levels of CB1 together with low levels of nNOS mRNA. Due to the low levels of CB1 expression and, thus, to the diffuse appearance of the signals in subcortical areas, it was not always possible to determine single CB1-expressing cells with the same high precision as in cortical regions. However, in dorsolateral caudate putamen, one of the two regions

where an altered CB1 expression was observed in the nNOS-KO as compared to the WT controls, an indicative evaluation of coexpression was possible. In the striatum, nNOS is present in scattered cells containing generally high levels of mRNA. In comparison, CB1 is expressed at low and uniform levels in the majority of medium-spiny neurons of the dorsolateral caudate putamen (Marsicano & Lutz, 1999; Chapter 2). An approximate numerical evaluation of nNOS/CB1 coexpressing cells revealed that about 50% of nNOS-positive cells also express low levels of CB1 mRNA (Fig. 3.3C). Due to the low levels of both CB1 and nNOS mRNA in the ventromedial hypothalamus, it was not possible to evaluate numerically the coexpression in this area using the double *in situ* technique.

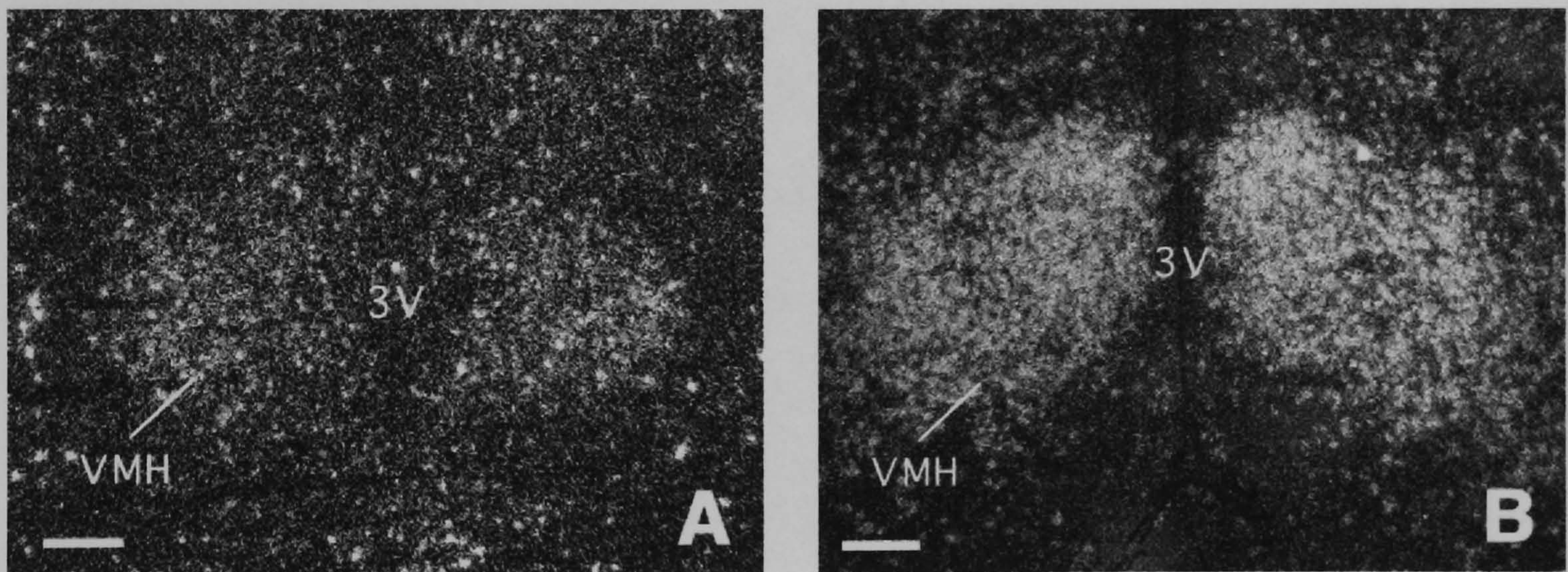


Fig. 3.4. Dark-field micrographs of parallel coronal sections from a WT mouse showing the expression of nNOS (A) and CB1 (B) in ventromedial hypothalamus. Note the similar distribution pattern of the two transcripts. Abbreviations: 3V, third ventricle; VMH, ventromedial hypothalamus. Scale bars: 100 μ m.

However, parallel sections hybridized either with radioactive riboprobes for CB1 or nNOS revealed a similar pattern of expression in this brain area (Fig 3.4). This observation allowed to conclude that in the mouse ventromedial hypothalamus, nNOS-expressing cells contain also CB1 mRNA at a rather high levels.

3.4 Discussion

This study aimed to investigate whether the nitric oxide pathway is involved in mediating Δ^9 -THC-induced central nervous system effects. Thus, the effects of Δ^9 -THC were examined in mice with targeted disruption of the neuronal nitric oxide synthase (nNOS) gene and in wild-type controls. Intraperitoneal injection of 10 mg/kg Δ^9 -THC resulted in the same increase of the hot plate latencies in both genotypes, indicating that Δ^9 -THC-mediated antinociceptive effects do not require nNOS. In contrast, a significant Δ^9 -THC-induced decrease of body temperature and locomotor activity was observed only in the wild-type mice, while the nNOS-KO mice showed a markedly reduced response. Determination of the expression of CB1 by *in situ* hybridization revealed significantly lower levels of CB1 transcripts in the ventromedial hypothalamus and the caudate putamen of the nNOS knock-out animals than in WT controls. These two areas are known to be among the regions involved in thermoregulation and cannabinoid-induced decrease of locomotion, respectively. Moreover, in these areas, CB1 and nNOS mRNAs are expressed to a rather high levels in the same neurons.

3.4.1 Antinociceptive effects

The lack of differences in nociceptive response between the two genotypes observed in this study is surprising as it would be expected that Δ^9 -THC, which has been shown to reduce NMDA-receptor-mediated Ca^{2+} influx (Hampson et al., 1998b), also inhibits the production of NO and, thus, the NO-mediated increase of presynaptic release of glutamate, which in turn plays a major role in pain transmission (Zieglgänsberger & Tölle, 1993; Tölle et al., 1996). Other studies indicate that Δ^9 -THC-induced antinociception also involves systems such as the spinal noradrenergic and the kappa opioid systems, both of which do not play a role in other central effects of Δ^9 -THC. For example, intrathecal administration of the α_2 -noradrenergic antagonist yohimbine was shown to block the antinociceptive effects of intravenously applied Δ^9 -THC, but failed to

antagonize its cataleptic or hypothermic effects, indicating that involvement of the adrenergic system is relatively specific to antinociception (Lichtman & Martin, 1991). In addition, intrathecal or intracerebroventricular administration of the kappa antagonist nor-BNI (nor-binaltorphimine) was able to block the antinociceptive effects of intrathecally applied Δ^9 -THC, but again without affecting Δ^9 -THC-induced catalepsy or hypothermia (Martin & Lichtman, 1998). These results together with the present findings suggest that Δ^9 -THC-induced supraspinal antinociception involves transmitter systems different from the NO pathway.

3.4.2 Effects on thermoregulation and locomotion

The results of pharmacological studies investigating the central role of the NO pathway in thermoregulation and locomotion are still contradictory (Scammell et al., 1996; Gourine, 1995; Simon, 1998; Calignano et al., 1997; Sandi et al., 1995; Johansson et al., 1997). However, the behavioural results described here clearly show that nNOS is required for Δ^9 -THC-induced decrease of body temperature and locomotor activity.

Several hypotheses can be put forward to explain the lack of effect of Δ^9 -THC in thermoregulation and locomotion in nNOS-KO mice. A very recent report (Nava et al., 2000b) showed that in rats the hypothermic effect of Δ^9 -THC is mediated by a coactivation of CB1 and dopamine D2-like receptors. Blockade of either CB1 or D2 completely abolished the hypothermic effects of Δ^9 -THC. The authors discuss that this effect could be attributed to the ability of Δ^9 -THC *in vivo* to induce the release of dopamine (Chen *et al.*, 1990a, b) and to activate dopamine neurons (Diana *et al.*, 1998; Gessa et al., 1998), which would consequently lead to a simultaneous activation of CB1 and D2-like receptors. Recently, Glass and Felder (1997) showed that in striatal neurons costimulation of CB1 and D2-like receptors leads to an accumulation of cAMP, in contrast to the decrease normally observed upon activation of either receptor alone. In neuroblastoma cells (Inada et al., 1998), cAMP accumulation was shown to stimulate

NO production. Dopamine D2 receptors are expressed in the striatum and in the ventromedial hypothalamus (Weiner et al., 1991) and are also colocalized with CB1 to a considerable extent at least in the dorsolateral caudate putamen (Hermann, Marsicano & Lutz, unpublished results). CB1 is expressed in approximately 50% of nNOS-positive cells in dorsolateral caudate putamen and in ventromedial hypothalamus a considerable number of neurons express both transcripts. Although triple colocalization studies would be required, it seems possible that CB1 and D2 receptors are coexpressed with nNOS in the same cell.

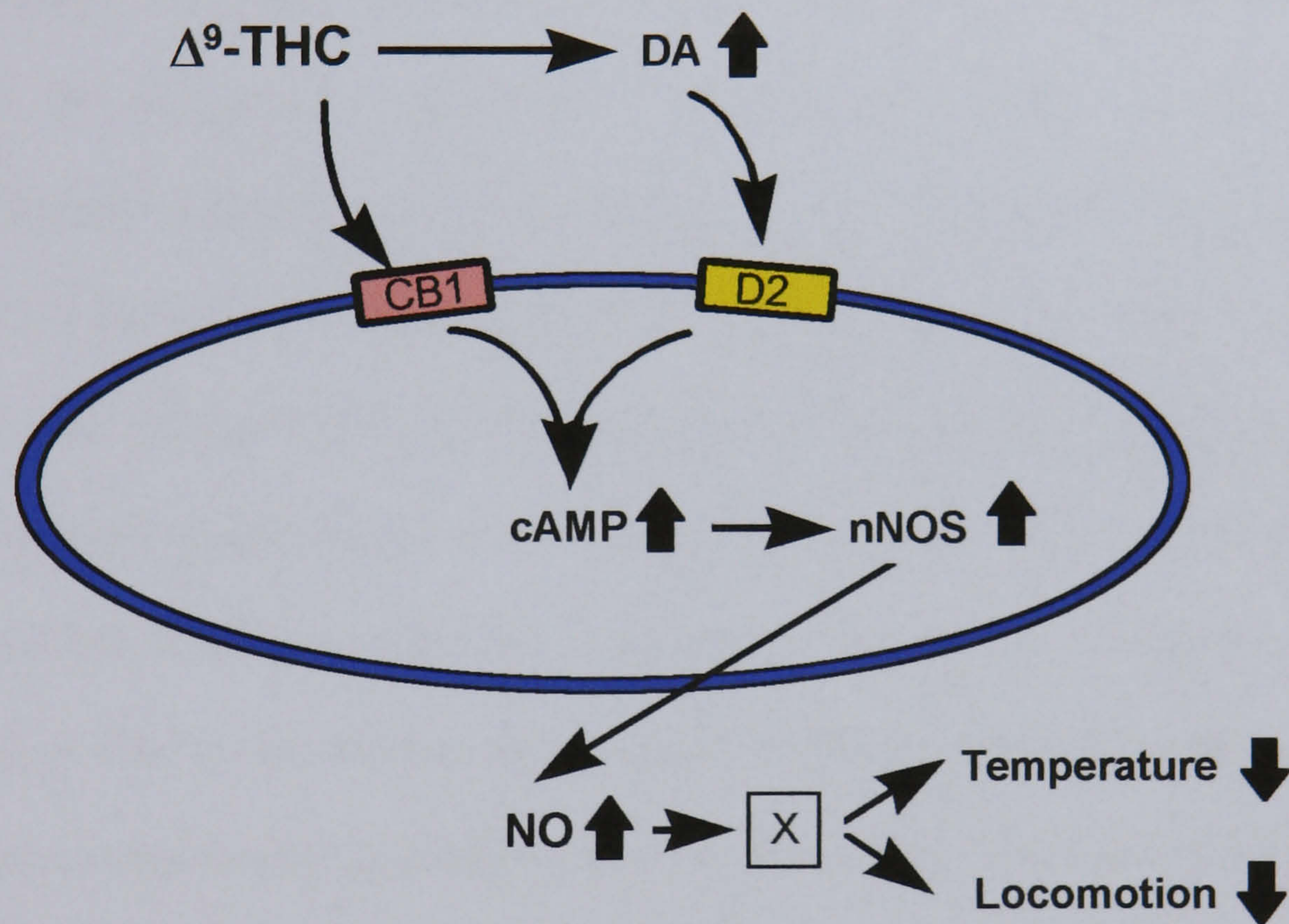


Fig. 3.5. Schematic representation of a putative mechanism through which Δ^9 -THC might exert its effects on thermoregulation and locomotor activity, involving the nNOS pathway. Δ^9 -THC administration would induce the release of dopamine (DA). Δ^9 -THC and DA would simultaneously or sequentially stimulate CB1 and D2 receptors, respectively, provoking an accumulation of cyclic adenosin monophosphate (cAMP). cAMP accumulation would lead to an activation of nNOS with a release of NO, which would exert the decreases in temperature and/or locomotion, through a still unknown mechanisms (X).

Taken together, as depicted in Fig. 3.5, these observations speak in favour of the following cascade of events: coactivation of CB1 and D2-like receptors on the same cell may result in an accumulation of cAMP with a subsequent increase of nNOS activity and

an enhanced release of NO, which would finally exert the hypothermic and, possibly, also the locomotor effects of Δ^9 -THC. This interpretation of the results, however, appears to be inconsistent with the report of Thorat and Bhargava (1994). These authors found that pretreatment of mice with the NOS inhibitor NG-monomethyl-L-arginine (L-NMMA) did not change analgesic or hypothermic effects of Δ^9 -THC. Such an apparent discrepancy could be explained by intrinsic differences between pharmacological and genetic approaches. It is possible that the treatment of animals with L-NMMA is not sufficient to completely block the stimulation by Δ^9 -THC. Alternatively, the disruption of the nNOS gene could lead to profound alterations, such as defective brain development, or/and to the disruption of downstream pathways that cannot be achieved by a pharmacological treatment with nNOS inhibitors. One example of a long-term effect caused by the disruption of the nNOS gene is our observation of decreased levels of CB1 mRNA in the striatum and the ventromedial hypothalamus in the knock-out mice. Alternatively or complementary to the explanation depicted in Fig. 3.5, this change in CB1 expression could also serve as a molecular mechanism to explain the decreased effects of Δ^9 -THC in locomotion and thermoregulation in nNOS-KO mice (Fig. 3.6). Investigations using human neuroblastoma, lymphoma and endothelial cells have revealed that anandamide is rapidly taken up by a high affinity transporter which can be activated by NO (Maccarrone et al., 1998, 2000). Within the cell, anandamide is then hydrolyzed to arachidonate and ethanolamine by the fatty acid amide hydrolase (FAAH) (Mechoulam et al., 1998; Di Marzo & Deutsch, 1998). In this context, it could be hypothesized that the lack of NO in the nNOS-KO mice may lead to a decreased re-uptake of endogenous anandamide and, thus, to an increased extracellular concentration. High levels of extracellular anandamide may in turn downregulate CB1 expression in selected areas such as the caudate putamen, as it is also described for long-term treatment of rats with Δ^9 -THC (Corchero *et al.*, 1999; Zhuang et al., 1998). Finally,

decreased levels of CB1 would lead to a reduced responsiveness to Δ^9 -THC as observed in this study (Fig. 3.6).

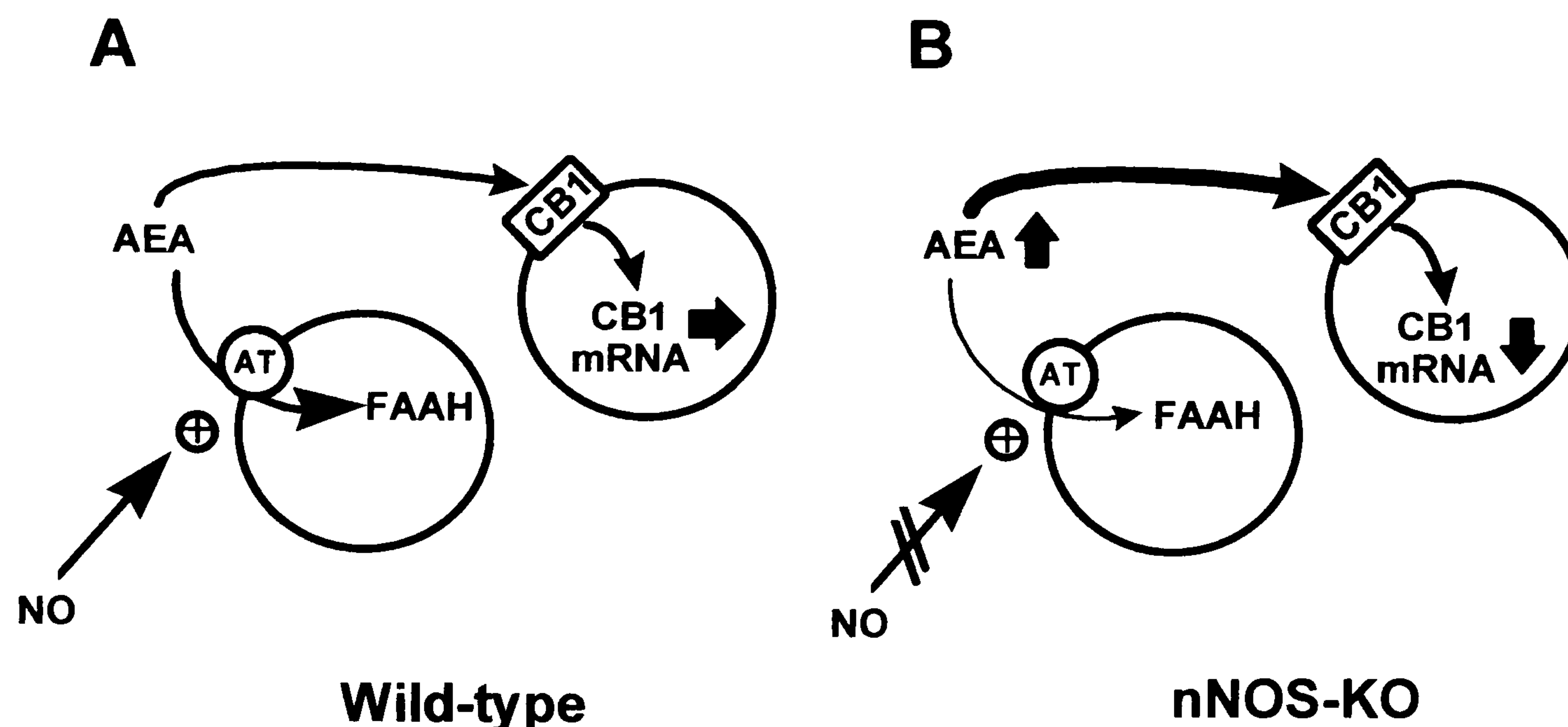


Fig. 3.6. Schematic representation of a putative mechanism through which the lacking of nNOS might lead to a decrease in CB1 mRNA expression and, thus, a decreased responsiveness to Δ^9 -THC in selected areas. A, Wild type situation: NO is able to activate the anandamide transporter (AT), thus contributing to the equilibrium between extracellular anandamide (AEA) and intracellular uptake and degradation through the enzyme fatty acid amide hydrolase (FAAH). Extracellular AEA binds to CB1 receptors in an equilibrate fashion and thus the turnover of CB1 mRNA is maintained physiologically constant. B, nNOS knock out situation: the lack of a functioning nNOS decreases the levels of NO and thus also the positive drive onto AT. Therefore extracellular levels of AEA are free to overstimulate CB1 receptors, with the consequent alteration of the receptor mRNA turnover and a decrease of CB1 mRNA synthesis. Decreased levels of CB1 would, in turn, account for the decreased activity of Δ^9 -THC in selected brain areas related to locomotion and thermoregulation. For clarity, the processes are depicted in different cells, but it is possible that they happen in the same cell.

Another mechanism explaining the observed down-regulation of CB1 can also be put forward. NO-responsive elements were characterized in the promoter of the tumor necrosis factor alpha gene and were found to be Sp1 binding sites (Wang et al., 1999). As the mouse CB1 gene also contains several Sp1 binding sites (Marsicano & Lutz, unpublished results), the down-regulation of CB1 mRNA levels could also be mediated by the reduced transcriptional activation of the CB1 gene due to reduced stimulation by NO. In other terms, the changed Δ^9 -THC responsiveness observed in the nNOS-KO

mice could then mainly or partly be due to the reduced levels of CB1 transcripts in the respective brain regions, i.e. in the ventromedial hypothalamus and in the dorsolateral caudate putamen.

In conclusion, the behavioural and molecular findings described in this Chapter clearly suggest that the nitric oxide pathway is involved in some of the central effects of Δ^9 -THC including hypothermia and decrease of locomotor activity. In addition, it appears that this transmitter system plays only a minor role in Δ^9 -THC-induced supraspinal antinociception.

In Chapter 2 and in the present Chapter, CB1 was analysed in some of its neuroanatomical and functional aspects. However, the cannabinoid system represent a potentially very important and complex neuromodulatory apparatus that is likely to interact with many neuronal systems in the brain. CB1-deficient mice could represent very powerful tools to gain deeper insights into the specific functions of the cannabinoid system in different brain activities. For this reason, a genetic approach was carried out to understand the functions of CB1 during the preparation of this Thesis. Next Chapter will describe an innovative gene targeting approach to enable us obtain a spatio-temporal specific disruption of CB1.

Chapter 4

Generation of mouse CB1 mutants

4.1 Introduction

During the last decade, targeted mutagenesis in mice has become one of the most powerful tools to study gene function. The potential to disrupt or modify in principle any gene of interest in the mouse genome has led to fundamental discoveries in all fields of mammalian biology, ranging from embryonic development to the generation of animal models for human diseases. A milestone in the development of the gene targeting technology was the establishment of totipotent cell lines (embryonic stem cells, ESCs) derived from mouse blastocysts (Bradley et al., 1984). If kept in appropriate conditions, these cells can contribute to all cell types of the body including germ cells. The second hallmark was the observation by Capecchi and colleagues (Folger et al., 1982) that mammalian somatic cells possess the enzymatic machinery for efficiently mediating homologous recombination between newly introduced, non-replicating DNA molecules. Both, homologous recombination and the availability of embryonic stem cells led to the first gene disruption experiments (Thomas & Capecchi, 1987; Doetschman et al., 1987), now currently referred to as gene "knock-out".

When this approach became available for the scientific community, hope arose to study and understand protein functions in the entire animal, thus avoiding many of the limitations of *in vitro* models. Also for behavioural studies, this technology appeared to be suitable to understand the basic mechanisms underlying highly complex brain functions, such as learning and memory, cognition and pain perception. Indeed, in the last 10 years, enormous progress has been made in the study of higher brain functions by using this technology. As an example, the study of learning and memory in gene targeted mutant animals has led to the discovery of important key mechanisms by which the brain is able to select, to store and to recall information (Silva et al., 1992; Grant et al., 1992).

Despite the progress enabled by this new technology, some intrinsic caveats and limitations became soon evident.

1. Lack of temporal restriction. In a "conventional" gene targeting experiment, the gene of interest is disrupted in every cell of the animal; thus, it is inactivated both during development and adult life in all tissues. However, many proteins have different functions in various stages and tissues, e.g. early functions, essential for the normal embryonic growth and development and later functions in the adult, participating in molecular mechanisms to control the physiological status of the organism, including specific behaviours. Thus, in a "conventional" knock-out, we might provoke general developmental defects that mask the function(s) of a gene in the adult.

2. Lack of spatial selectivity. The central nervous system is highly complex. Different anatomical regions and different cell types in the same regions differentially contribute to various physiological functions. On the other hand, particular brain functions, such as behaviour, are the result of coordinate interactions between different anatomical regions and cell types. In most cases, proteins are expressed in many anatomical regions and cell types, very often exerting different tasks. Therefore, in the attempt to understand the contribution of a given gene product to particular CNS functions, it is essential to "dissociate" this contribution into its anatomical and cellular aspects. When a gene function is disrupted by "conventional" gene targeting, all body cells will carry this modification. Therefore, it is difficult, if not impossible, to relate the protein function to particular anatomical brain regions and cell types.

3. Compensatory mechanisms. Mammalian cells in general, and neuronal systems in particular, are equipped with finely regulated control systems which help to overcome naturally occurring imbalances. Thus, the disruption of a gene at early stages of development could induce compensatory mechanisms which could mask important functions of the gene in the adult physiology. Such mechanisms could range from the simple compensatory

overexpression of other related proteins with similar functions, to the misexpression of non-related genes, finally leading to a possible phenotype masking the function of the targeted gene and resulting in an interpretation of gene function that is only partially correct (for a review, see Gerlai, 2000).

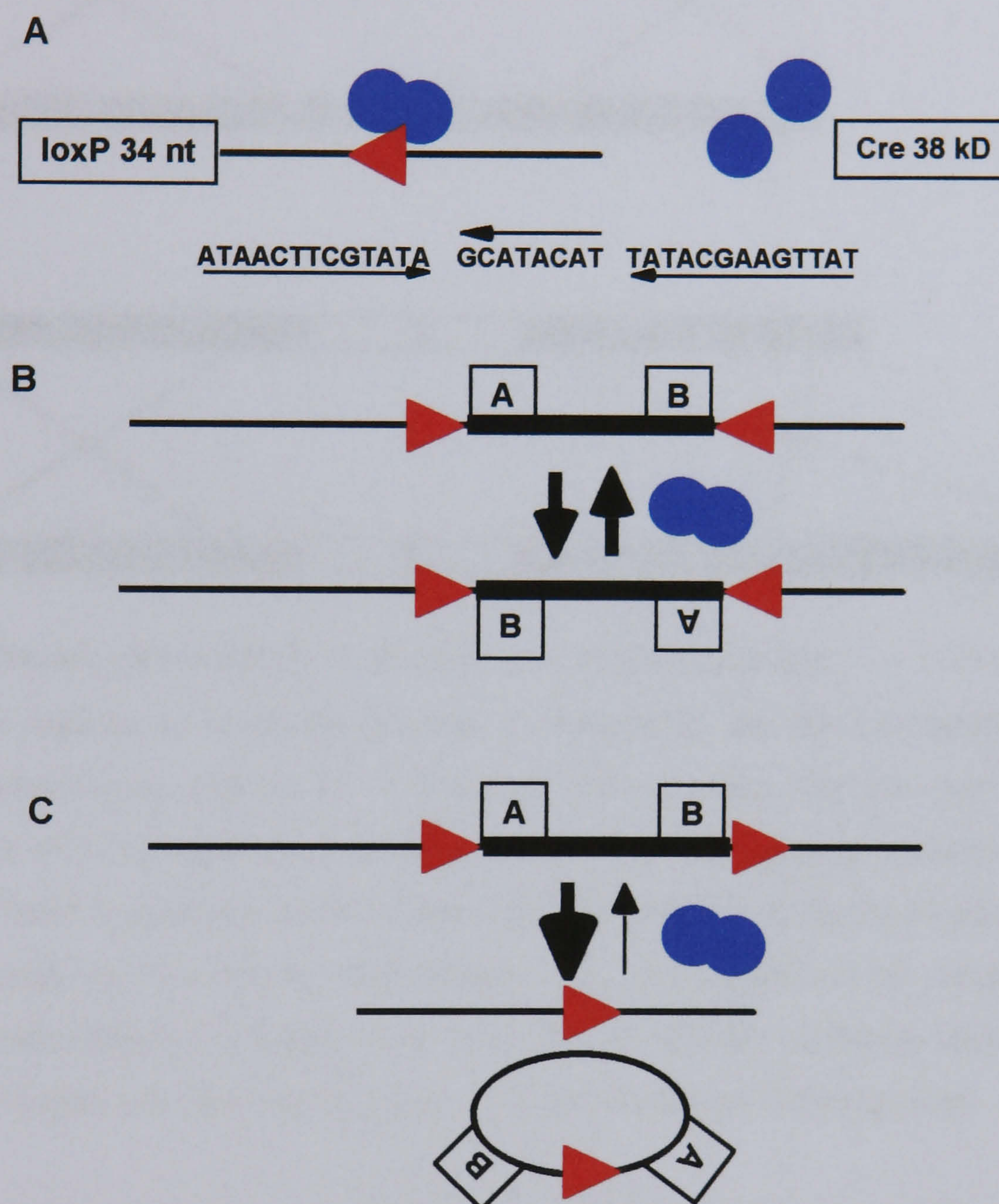


Fig. 4.1. Schematic representation of the Cre/loxP system. A. Graphical representation of loxP site (red triangle), Cre recombinase (blue circle) and nucleotide sequence of the loxP site. LoxP consists of two sequences forming a palindrome, which is separated by a non-palindromic nucleotide stretch. Orientation of the loxP site is given by the orientation of this core sequence. Two molecules of Cre bind to one loxP site (Mack et al., 1992). B. When loxP sites are oriented "head-to-head", Cre recombinase catalyzes an inversion of the sequence between the two loxP sites. C. When loxP sites are oriented "head-to-tail", Cre recombinase catalyzes an excision of the interposed sequence.

Therefore, during the last years, great efforts have been made to improve the gene targeting methodology in order to reduce the intrinsic problems of the "conventional" knock-out, and to obtain disruption or modification of targeted genes in a more selective way. This

new technology is commonly referred to as "conditional" gene targeting, using the Cre/loxP system (Sauer & Henderson, 1989).

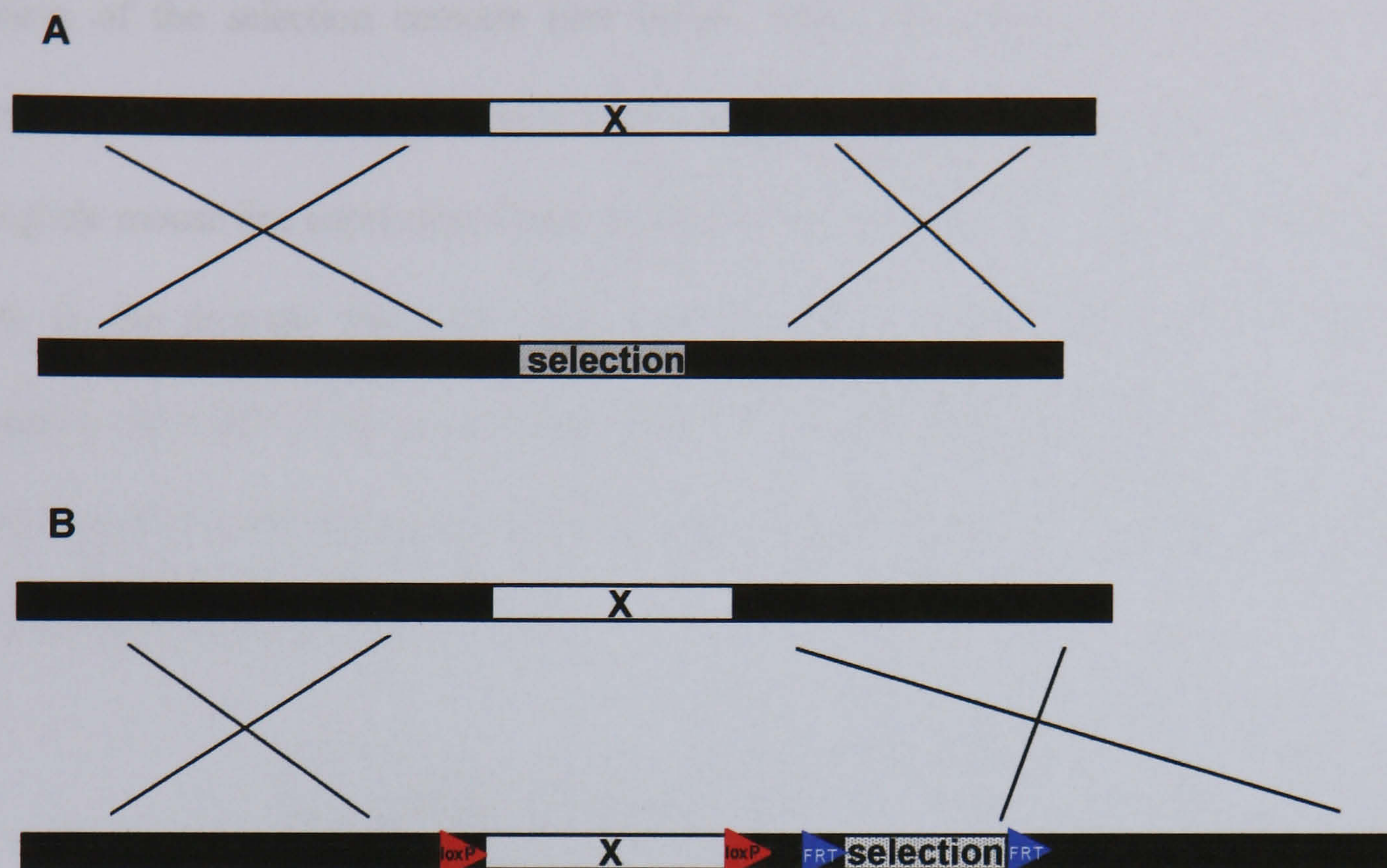


Fig. 4.2. Schematic representation of different gene targeting strategies. A. "Conventional" gene targeting. The sequence to be deleted (X, top) is displaced by the selection cassette (usually an antibiotic resistance gene, bottom). B. "Conditional" gene targeting. The wild type gene (top) is conserved, but two loxP sites and a selection cassette from the targeting construct (bottom) are introduced. In order to guarantee the completely wild type expression of the floxed gene, the selection cassette is flanked by two different recombination sites (FRT), that will be excised by another recombinase, called flipase (see below, same paragraph). X, sequence of interest; selection, selection cassette; red triangles, loxP sites; blue triangles, FRT sites; black bars, homology arms.

Cre recombinase is a P1 bacteriophage-derived enzyme that is able to mediate site-specific recombination between a 34 bp-long sequences referred to as loxP (locus of crossover x in P1) (Sternberg & Hamilton, 1981). The loxP site consists of two 13 bp-long repeats oriented head-to-head and forming a palindrome, which is interrupted by an 8 bp-long non-palindromic sequence. This sequence gives an intrinsic orientation to the loxP site (Fig. 4.1).

To generate a "conditional" knock-out with the Cre/loxP system, two components are required. First, by homologous recombination in ES cells, two loxP sites are introduced 5' and 3' to the gene of interest (Fig. 4.2). Second, cells containing the correctly recombined

locus are then used to generate a mouse line expressing the "floxed" (i.e. flanked by two loxP sites) sequences of interest. Given the shortness of the loxP sequences, and provided the excision of the selection cassette (see below, same paragraph) this mouse line is still expressing the gene of interest in a wild type manner. This line is then crossed with a transgenic mouse line expressing Cre-recombinase in a defined tissue and/or at a distinct time point. In the progeny mice, Cre will recognize the two loxP sites and will excise the sequences between these sites. Depending on the regulatory sequences that drive the expression of Cre, the excision of the sequence of interest will be "conditioned" in a temporal and/or spatial way (Fig. 4.3).

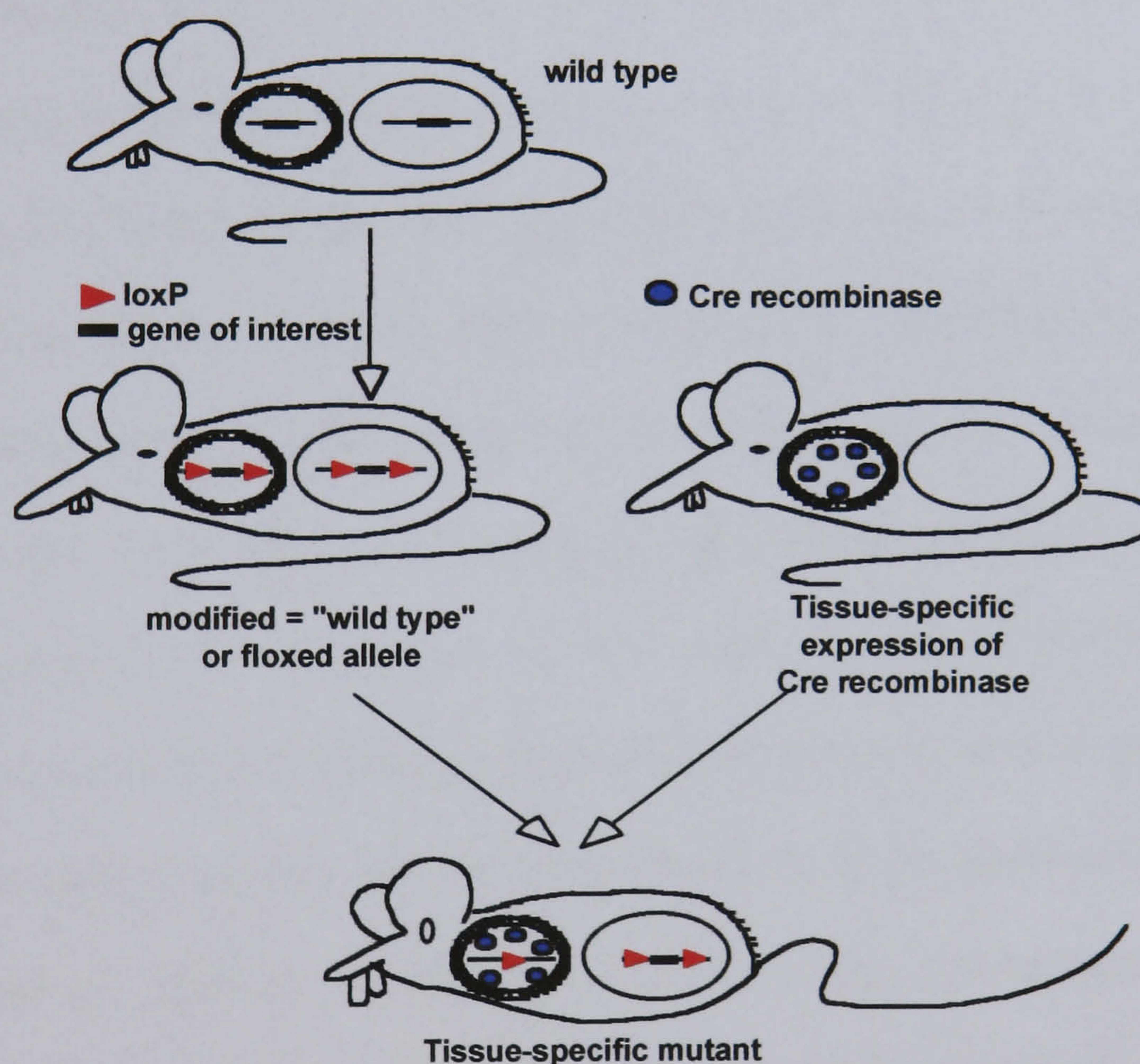


Fig. 4.3. Schematic representation of a "conditional" gene targeting using the Cre/loxP system. Red triangles are loxP sites; blue circle is Cre recombinase; black box is the gene of interest.

CB1 is expressed in several different regions of the CNS and, as shown in Chapter 2 and 3, in different cell populations. It is also expressed, even though at lower levels, in peripheral tissues (e.g. Shire et al., 1995) and at high levels during the entire embryonic development (Buckley et al., 1998; Paria et al., 1995; Berrendero et al., 1998). Therefore, a "conventional" gene knock-out might not give conclusive insights into the roles of CB1 in the

adult brain physiology. Indeed, despite the valuable information gained, the published CB1 mutants obtained by "conventional" gene targeting approaches reveal phenotypic aspects whose origin might be partially due to the lack of temporal and/or spatial restriction of the gene deletion. As an example, it cannot be excluded that the lack of CB1 expression during embryonic development of the mutants is among the causes leading to the defects described by Zimmer et al. (1999) in adult animals. These mice showed hypomotility, hypoalgesia and premature death. Both hypomotility and hypoalgesia are surprising, considering the similar effects caused by pharmacological treatment with CB1 agonists (Chaperon & Thiébot, 1999). Locomotion defects are likely due to alterations in gene expression in striatal cells, where the transcripts encoding for the opioid peptides dynorphin and enkephalin are present at higher levels in mutant animals as compared to wild type controls (Steiner et al., 1999). As it was shown that cannabinoid treatment during pregnancy can increase the adult expression of opioid peptides, decrease opioid receptors and increase the morphine self-administration in adult offspring (Vela et al., 1998; Corchero et al., 1998; Manzanares et al., 1999), it cannot be excluded that the defects observed in adult CB1 mutant animals are due to alterations occurring during *in utero* development. In other words, it is possible that the disruption of a functional cross-talk between cannabinoid and opioid system *in utero* is one of the principal causes of the adult phenotype observed in the mutants, thus, the function of CB1 in the adult brain physiology might be masked by alterations during development (Gerlai, 2000). Embryonic deficits could also account for the early mortality described in this mutant line (Zimmer et al., 1999). At birth, the expected Mendelian distribution is not completely reached, likely indicating a certain rate of embryonic lethality of CB1 null mutants. Furthermore, homozygous mice died without any obvious sign of disease in a higher incidence than heterozygous or wild type littermates, starting as early as 6-8 weeks after birth. Obviously, this might represent a major obstacle in the phenotypic analysis of the adult. In addition, the hypomotility of the mice described by Zimmer et al. (1999) might mask other

important phenotypic aspects of the mutants. As an example, the majority of learning and memory paradigms require the animal to accomplish tasks in which a correct locomotion system is necessary. It appears obvious that it is very difficult to test hypomotile mice in such paradigms. A spatially restricted deletion of CB1 in brain regions not directly involved in locomotor functions would presumably avoid this obstacle (see 4.4 "Discussion"). Also Ledent and colleagues (1999) described another mutant CB1 mouse line obtained by "conventional" gene targeting. In contrast to the results of Zimmer et al. (1999), these mutant mice did not present any evident phenotypes without pharmacological treatment. They were unresponsive to cannabinoid treatment and, interestingly, showed reduced sensitivity to addictive effects of opiates (Ledent et al. 1999). The apparent discrepancies between the phenotypes of these two mouse lines are likely to be caused by the different genetic background used during analysis. CB1-KOs from Ledent et al. (1999) were bred in the outbred CD1 strain, while the line of Zimmer et al. (1999) was back-crossed into the inbred C57BL/6J strain. It is possible that CB1 has different functions in adult brain physiology in these two mouse strains, but it appears also likely that the lack of CB1 causes different developmental alterations in different strains, thus, leading to different adult phenotypes. Generally, outbred strains possess a higher vigor than inbred strains, probably due to the lower homozygosity rate. Therefore, it could be suggested that inbred strains such as C57BL/6J have compensatory mechanisms during prenatal development insufficient to completely compensate the lack of CB1. On the other hand, the outbred CD1 strain may have a better compensatory machinery. Moreover, it is interesting to note that the alterations in gene expression described by Zimmer and colleagues (Steiner et al., 1999) also involve components of the endogenous opioid system, such as dynorphin and enkephalin. This observation, together with the above mentioned experiments showing that prenatal cannabinoid treatment causes profound alterations in the adult endogenous opioid signalling (Manzanares et al., 1999), might indicate a similar interaction between CB1 and opioid

system at prenatal stages also in the CB1-mutant mice described by Ledent et al. (1999). Therefore, it cannot be excluded that also the cause of the reduced sensitivity to the addictive effects of opiates as observed in the mice generated by Ledent et al. (1999) resides in the disruption of the functional cross-talk between CB1 and opioid system *in utero*, similarly to as hypothesized above for CB1 mutants in C57BL/6J background. These potential developmental effects of the disruption of CB1 were not analysed by the two groups. However, it appears possible that the phenotypes described by Zimmer et al. (1999) and Ledent et al. (1999) in adult mutant animals are only partially due to the lack of the gene at the moment of the analysis. Moreover, it is possible that the discrepant results obtained in the two different strains are due to a different compensatory potential to similar alterations occurring during *in utero* development. In other words, the hypoalgesia and hypolocomotion observed in the mutant mice from Zimmer et al. (1999) and the lack of morphine self-administration described in the mice of Ledent et al. (1999) cannot necessarily be attributed to specific functions of CB1 in the adult brain physiology.

As mentioned above, one of the aims of "conditional" gene targeting is to avoid any possible misinterpretation caused by "conventional" approaches, where compensatory mechanisms might mask the real function of a deleted gene. Moreover, while "conventional" gene targeting experiments do not have spatial resolution, the "conditional" approach can provide information about the specific region or cell type where CB1 function resides. Taken together, in order to analyze the physiological roles of CB1 in the adult mouse brain, a "conditional" gene targeting approach was chosen, with the hope to obtain information as precise as possible and not to mask the phenotypes by "compensatory" mechanisms.

The generation of a "floxed" mouse line is a time-consuming experiment. It is therefore important to be able to show the actual functioning of the system as soon as possible. The quickest way to demonstrate the Cre-mediated excision of the gene in the living animal is the generation of a "null mutant", by crossing the "floxed" mouse line with a so-called "Cre-

deleter" mouse line, in which Cre expression is driven by a constitutive promoter (Schwenk et al., 1995). By this way, the excision of the gene of interest is achieved in all body cells, including germ cells. Moreover, despite the fact that a "conventional" null mutation approach has the main limitations described above, e.g. for behavioural analyses, such mutants can constitute an useful tool for phenotypic analyses at cellular levels, such as investigations on primary neuronal cultures. CB1 null mutant mice (designated here as CBN) were generated in order to study the neuroprotective effects of cannabinoid compounds *in vitro* (Chapter 5). Another important prerequisite of the Cre/loxP approach is the perfect wild type expression of the "floxed" gene before the Cre-mediated excision. The targeting approach needs the presence of a selection gene to select for the recombined locus. Usually, the enzyme neomycin phosphotransferase "Neo") is used to confer to the cells the ability to survive in presence of the neomycin derivative geneticin (G418). Generally, the presence of such a "cassette" in the gene locus is not a problem in the "conventional" gene targeting approach, as the gene of interest is simply substituted by the selection cassette (Fig. 4.2A). In a "conditional" gene targeting approach, however, the presence of the "Neo" cassette might influence the expression of the targeted gene. Therefore, in the gene targeting construct described in this Chapter, the "Neo" cassette is flanked by two other recombination sites, named "FRT" (Flipase Recombination Target). These sites are recognized by another recombinase called "flipase" (Flp) (Landy, 1992). As Flp/FRT system is originally derived from yeast, which grow at 30°C, it is less efficient than Cre/loxP at 37° C. However, Flp/FRT still maintains a sufficient activity to allow a Flp-mediated excision in mammalian cells (Dymecki, 1996). In this thesis, it was decided to excise the "Neo" cassette by crossing the "floxed-Neo" CB1 mice with a "Flp-deleter" mouse line, which express Flp recombinase ubiquitously (Dymecki, 1996).

In this Chapter of my thesis I will describe the generation of the "floxed CB1" mouse line. Moreover, the *in vivo* functioning of the Cre-mediated excision of the "floxed" CB1 gene

will be shown by the generation of the CB1 null mutant (CBN). Further potential applications of the technology to the study of the cannabinoid system in the mouse physiology are also discussed.

4.2 Materials and methods

4.2.1 Molecular biology

Where not differently stated in the results section, these are the basic methods used during the work. Solutions are described as final concentration. Stock solutions were prepared according to Sambrook et al. (1989). DNA sequencing was performed by MWG Biotech (Germany). The plasmid pFTM-loxP, containing the 34 bp-long loxP site, was obtained from Dr. Theo Mantamadiotis, Heidelberg, Germany. The plasmid pSVpaZ11-PGK-Neo, containing the selection Neo cassette (neomycin phosphotransferase gene) under the control of the human PGK (phosphoglycerate kinase I) promoter and flanked by two FRT sites was a kind gift of Dr. Bernhard Lüscher, Zurich, Switzerland. This plasmid constitutes a derivative of pSVpaZ11, generated by Dr. Francis Stewart, Heidelberg, Germany. "Cre-deleter" mouse line is described in Schwenk et al. (1995) and was kindly provided by Prof. Rajewsky, Köln, Germany. "Flp-deleter" mouse line is described in Dymecki (1996) and was kindly provided by Dr. Susan Dymecki, Baltimore, U.S.A. Both these mouse lines were bred in a C57BL/6 genetic background.

4.2.1.1 Restriction digestions and ligations

Restriction enzymes and modification enzymes were purchased from New England BioLabs (NEB, USA). Digestions of plasmid DNA were carried out in the appropriate buffer at the recommended temperature for 1-5 hours. Enzyme units used were adjusted to the amount of DNA to be digested. When necessary, DNA fragments were blunted after digestion. Briefly, DNA digest (2-5 µg) was mixed in a total volume of 100 µl containing 1x T4 DNA polymerase buffer, 0.5 mg/ml BSA, 0.1 mM each of dATP, dCTP, dTTP, dGTP,

4.5 units of T4 DNA polymerase. The mixture was incubated 20 minutes at 11°C, and the enzyme was inactivated by adding 2 µl of 0.5 M EDTA. For ligations, DNA fragments and vectors were separated by gel electrophoresis (0.8-1.5% agarose in 1x TAE: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0, containing 0.1 µg/ml ethidium bromide) and purified using the Gel Extraction kit (Qiagen, Germany). Ligations were performed with T4 DNA ligase, in 10 µl of provided ligation buffer and with molar ratios of vector: insert ranging from 1:2 to 1:10. All plasmids generated in this thesis are derived from pBlueScript KS(-) (pKS, Stratagene, U.S.A.). Digestions of genomic DNA were performed after precipitation of 10-20 µg DNA from mouse tail or of the entire DNA content of a single well of a 96-well plate of DNA from ESC clones (see below). DNA pellets were resuspended in 30 µl of final digestion solution, containing 1x buffer, 100 µg/ml RNase A (NEB, USA) and 100 units of restriction enzyme. After 6-12 hours of incubation at the recommended temperature, spermidine at final concentration of 4 mM and 60 units of enzyme were added, and the reaction was further incubated at the same temperature overnight.

4.2.1.2 Polymerase Chain Reaction (PCR)

PCR reactions were performed in a final volume of 50 µl containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 pmol/µl of both forward and reverse primers, 1 µl of DNA template (50-500 ng), 1 µl of DNA polymerase. Taq polymerase was used for most of the reactions. When particularly precise amplification was needed, recombinant Pfu polymerase (Stratagene, USA) was used. All primers were designed to have a T_m of approximately 58°C. Thus, all PCR reactions were carried out in a Robocycler (Stratagene) with the following parameters: 1 cycle at 95°C for 5 minutes; 37 cycles at 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute; 1 cycle at 72°C for 5 minutes. Table 4.1 shows name, sequence, orientation and relative position of the primers designed and used in this chapter.

Name	Sequence	Orientation	Position
G1	5' - GTT GAG CCT GGC CTA ATC AAA - 3'	forward	- 30 bp from CB1 ATG
G2	5' - CCG ATG AGA CAA CAG ACT TCT - 3'	reverse	+ 1500 bp from CB1 ATG
G3	5' - GTT GAC CGA ACC TCT GTT TTC - 3'	forward	- 182 bp from CB1 ATG
G7	5' - GGA CTT GGC AGA CGA ATT GTA - 3'	forward	- 199 from putative intron donor site
G8	5' - CTA CCC CTG AAG GAA GTT AGA - 3'	reverse	+ 167 from CB1 ATG
G50	5' - GCT GTC TCT GGT CCT CTT AAA - 3'	forward	- 563 bp from CB1 ATG
G51	5' - GGT GTC ACC TCT GAA AAC AGA - 3'	reverse	- 150 bp from CB1 ATG
G52	5' - GAC TGT CAC CGT GCT TAC TTA - 3'	forward	+ 1539 bp from CB1 ATG
G53	5' - CTC CTG TAT GCC ATA GCT CTT - 3'	reverse	+ 1957 bp from CB1 ATG
G54	5' - CCT ACC CGG TAG AAT TAG CTT - 3'	reverse	+ 22 bp from 5' FRT site in FRT-PGK-Neo cassette

Table 4.1. PCR primers.

"Cre-deleter" mice were genotyped by PCR as described in Schwenk et al. (1995), using the primers B42 (5'- GAT CGC TGC CAG GAT ATA CG - 3') and B43 (5'- CAT CGC CAT CTT CCA GCA G - 3'), in the same conditions as described above. "Flipase-deleter" mice were genotyped as described in Dymecki (1996), using the primers B34 (5'- GTG GAT CGA TCC TAC CCC TTG CG - 3') and B35 (5'-GGT CCA ACT GCA GCC CAA GCT TCC - 3'), in the following conditions: 1 cycle at 95°C for 5 minutes, 70°C for 3 minutes and 72°C for 5 minutes; 34 cycles at 90°C for 1 minute, 70°C for 1 minute, 72°C for 1 minute; 1 cycle at 72°C for 10 minutes.

4.2.1.3 Transformation and growth of bacteria, bacterial colony lift and plasmid purification

Bacteria were transformed with a few ng of plasmid DNA, or with 5 µl of ligation reaction. Competent DH5α cells were thawed on ice. Plasmid DNA or ligation reaction was added to 200 µl of bacterial suspension and incubated for 30 minutes on ice. After 1 minute and 30 seconds at 42°C, 800 µl of SOC buffer (20 g/l bactotryptone, 5 g/l yeast extract, 0.5 g/l NaCl, 20 mM glucose) were added and cells were shaken for 30 minutes at 37°C. Finally, bacteria were plated onto Luria Broth (LB)-agar plates containing 100 µg/ml

ampicillin. In some cases, colony lifts were necessary to find positive clones. The procedure for bacterial colony lifts was identical as for the phage plaque lifts (see below, 4.2.1.4 "Growth of genomic DNA phage library and phage plaques lift"), with the only differences that one single Hybond NX membrane (Amersham, USA) was used for each plate and that, after transfer, bacterial plates were reincubated at 37°C for 2-4 hours in order to rescue the colonies. Mini plasmid purifications were carried out by growing single bacterial colonies in 2 ml of LB overnight. 1 ml of the culture was then centrifuged and the cell pellet was lysed in 300 μ l of TENS buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA, 0.1 M NaOH, 0.5% SDS). After 5 minutes of lysis, 150 μ l of 3 M Sodium acetate pH 5.2 were added and, after vortexing, the lysate was incubated on ice for 5 minutes. Then, the preparation was centrifuged at 16,000 xg for 10 minutes at 4°C. 400 μ l of the supernatant was collected in a fresh tube containing 900 μ l of 100% ethanol. DNA was precipitated for 5 minutes at 4°C, and centrifuged at 16,000 xg for 5 minutes. The DNA pellet was washed with 70% ethanol, air-dried and resuspended in 30 μ l of TE buffer (10 mM Tris/HCl pH 7.5, 1 mM EDTA), containing 100 μ g/ml RNase A.

4.2.1.4 Growth of genomic DNA phage library and phage plaques lift

A lambda DASHII genomic library constructed from E14 embryonic stem cells partial Sau3A genomic digestion (Kühn et al., 1991) was screened to isolate DNA fragments containing the CB1 locus. A single colony of the host e. coli LE392 was inoculated in 50 ml of LB, supplemented with 0.2% maltose and 10 mM MgSO₄. Cells were incubated overnight at 30°C and were then centrifuged at 2000 rpm. Bacterial pellet was gently resuspended in 30 ml of 10 mM MgSO₄. From this point on, bacteria were kept on ice. The optical density at 600 nm (OD₆₀₀) of bacterial suspension was measured and the cells were diluted in 10 mM MgSO₄ to OD₆₀₀ = 0.5. NZY agar plates (22x22 cm for primary screening or 9 cm diameter for further screenings) were prewarmed at 43°C. NZY agar plates contain 5 g/l NaCl, 2 g/l MgSO₄-7H₂O, 5 g/l yeast extract (LIFE Technology, U.S.A.), 10 g/l NZ Amine (casein

hydrolysate, Sigma, U.S.A.), and 1.5% Difco bacto agar. Absorption of phages onto bacteria was obtained by incubating (15 minutes at 37°C) 1.2 ml of host bacteria ($OD_{600} = 0.5$) with 900 μ l of phages diluted in SM buffer (primary screenings), or 200 μ l of host bacteria with 100 μ l of phages (further screenings). SM buffer consists of 5.8 g/l NaCl, 2 g/l $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, and 0.01% gelatin, pH 7.5. As determined by preliminary titration experiments, 100,000 plaque forming units (pfus) were used for primary screening, 10,000 pfus for secondary, 1,000 pfus for tertiary and 100 pfus for quarterly. Top agar (as NZY agar plates, but containing 0.7% agarose instead of Difco bacto agar) was melted in microwave and cooled down in water bath to 48°C. 25 ml (primary screenings) or 2.5 ml (other screenings) of top agar were added to the phage/bacteria mixture, quickly mixed and immediately poured onto NZY agar plates that were prewarmed at 43°C. After solidification of top agar/phage/bacteria mixture, plates were incubated overnight at 37°C. After growth of phages, appearing as plaques in the layer of bacteria, plates were chilled at 4°C. Hybond NX membranes (Amersham, USA) of the appropriate size were carefully placed onto the surface of the plates and were let soak for 2 minutes. Asymmetrical holes were made onto the membrane with a syringe needle, in order to know the relative position of plaques. After peeling the first membrane, a second membrane was put onto the plate surface for a second transfer of 5 minutes. Membranes with phage plaques uppermost were placed onto a series of sheets of 3MM filter paper soaked with denaturing and neutralizing solution, for 5 minutes and twice for 3 minutes, respectively. Denaturing solution contains 1.5 M NaCl, and 0.5 M NaOH. Neutralizing solution contains 1.5 M NaCl, and 0.5 M Tris-HCl pH 7.4. After the second neutralizing step, membranes were vigorously washed in 2x SSC (20x SSC is 3 M NaCl, 0.3 M Na_3 citrate, pH 7.0) to remove protein debris and were then air-dried. DNA was fixed to the membrane by UV crosslinking in a Stratalinker (Stratagene, USA).

4.2.1.5 Isolation of genomic DNA

4.2.1.5.1 DNA preparation from mouse tails

About 0.5-1 cm of a mouse tail was cut and put into 700 μ l of tail lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS). 40 μ l of 10 mg/ml Proteinase K stock were freshly added and tails were incubated overnight at 56°C. Then, 300 μ l of saturated NaCl (7 M) were added, and the mixture was shaken for 5 minutes. After centrifugation at room temperature at 16,000 xg for 5 minutes, 750 μ l of the supernatant were transferred to a fresh tube, containing 500 μ l isopropanol. After mixing by inversion and centrifugation as above, the supernatant was removed, and the DNA pellet was washed once in 70% ethanol and air-dried for 10-20 minutes at room temperature. Finally, the DNA pellet was resuspended in 150-300 μ l of TE and stored at -20°C.

4.2.1.5.2 DNA preparation from embryonic stem cells

G418 resistant embryonic stem cells clones (see 4.2.2.3 "Electroporation of ESCs, antibiotic selection, picking and expansion of resistant clones") were grown on gelatin-coated 96-wells plates until they reached complete confluence. Then, cells were rinsed twice with PBS (139.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄-H₂O, 1.8 mM KH₂PO₄, pH 7.4) and 50 μ l of ESC lysis buffer (10 mM Tris HCl, 10 mM EDTA, 10 mM NaCl, 0.5% Sarcosyl and 1 mg/ml Proteinase K freshly supplemented) was added. Plates were incubated overnight at 50°C in a sealed humid chamber. Then, plates were spun 1 minute at 2500 rpm, and 100 μ l of a freshly prepared mixture containing 150 μ l of 5 M NaCl dissolved in 10 ml of 100% ethanol were added to precipitate nucleic acids. Plates were shaken at room temperature for 30 minutes and were then spun again at 2500 rpm. Supernatant was discarded, and precipitated nucleic acids were rinsed three times with 150 μ l of 70% ethanol. After the final wash, precipitated nucleic acids were either left in 70% ethanol and stored at -20°C, or air-dried for 10 minutes and resuspended in digestion solution (see above, 4.2.1.1 "Restriction digestion and ligations") for Southern blot analysis or in TE buffer for PCR analysis.

4.2.1.6 Southern blotting

4.2.1.6.1 Electrophoresis and transfer

Digested genomic, phage or plasmid DNA was electrophoresed in 0.8-1.2% agarose gels. The gels were then photographed on a UV transilluminator and then shaken 10 minutes in a solution of 0.25% HCl. After rinsing with distilled water, the gels were shaken 30 minutes in denaturation buffer and then, 30 minutes in neutralizing buffer (see above, 4.2.1.4 "Growth of genomic DNA phage library and phage plaques lift", for composition of the buffers). Capillary blot was performed in 10x SSC, as described in Sambrook et al. (1989), to transfer DNA onto Hybond NX filters (Amersham, USA). Gels with genomic and phage DNA were blotted overnight, whereas gels containing plasmid DNA were blotted with three different filters for 10 minutes, 5 minutes, and overnight, respectively. This procedure allows to hybridize the same plasmid digestions with three different probes at the same time.

4.2.1.6.2 Hybridization

Membranes containing either digested genomic, phage or plasmid digested DNA after blotting, or bacterial colonies or phage plaques after membrane lifts, were pre-incubated for 2 hours at 65°C in 20 ml of pre-warmed hybridization buffer (0.5 M Na-phosphate buffer pH 7.2, 7% SDS, 10 mM EDTA). Probe labeling was performed using the Random Primers DNA Labeling System kit (LIFE, USA) following the manufacturer's recommendations. Briefly, 100 ng of DNA fragment was denatured 5 minutes at 95°C in 10 µl of H₂O. Then, 15 µl of Random Primer Mix, 6 µl of a mixture containing 0.5 mM of each dATP, dCTP, dGTP, 1 µl of Klenow enzyme (3 units) and 5 µl ³²P-dCTP (3000 Ci/mmol; 50 mCi, Amersham, U.S.A.) were mixed with the denatured fragment in a final volume of 50 µl. Labeling reaction was carried out at 37°C for 30-60 minutes. Radioactive probe was purified using MicroSpin Column S-300HR (Pharmacia, Germany), following manufacturer's recommendations. Indicative evaluation of incorporation rate was performed with a hand radioactivity counter, comparing the amount of radioactivity in the MicroSpin Column (unincorporated) to the

amount in the eluted fraction (incorporated). Eluted probes were denatured for 5 minutes at 95°C and chilled on ice. After preincubation of the membrane, hybridization buffer was substituted with 10 ml of fresh prewarmed buffer and the radioactive probe was added. Hybridization was carried out at 65°C overnight. After hybridization, the hybridization mixture was frozen at -20°C (re-used up to three times, but not for genomic DNA), and membranes were subjected to stringent washes according to the following protocol: brief rinse in 50 ml of 2x SSC/0.1% SDS at room temperature, 10 minutes in 50 ml of 2x SSC/0.1% SDS at 65°C, twice for 10 minutes in 0.1x SSC/0.1% SDS at 65°C. Wet membranes were sealed in plastic bags and exposed for 3 hours to 3 days to autoradiographic films (DuPont, U.S.A.), using markers for orientation of the film to the membrane (Stratagene, U.S.A.).

4.2.1.7 Screening of genomic DNA phage library and large-scale preparation of lambda phages

After hybridization with the probe CB1HII, corresponding to the CB1 ORF (Fig. 4.4), only signals present on both membranes from the same plate were considered as corresponding to positive plaques. After orienting of the autoradiographic film with membranes and with plates, positive plaques were picked from the agar plate. In primary screenings, the wider end of a sterile Pasteur pipette was used to pick the surrounding area of positive plaques, and the piece of agar containing the phages was blown into an Eppendorf tube containing 1 ml of SM buffer. Phages were eluted by cutting the agar piece in small fragments and incubating at 4°C overnight with gentle shaking. For storage of phages, 2 drops of chloroform were added to the eluted SM buffer. For secondary and tertiary screenings, the eluted phages were treated as described above (see "4.2.1.4 Growth of genomic DNA phage library and phage plaques lift"), with the only differences that higher phage dilutions were plated out onto smaller plates and that smaller positive areas were picked from the plates. Finally, after 3 or 4 rounds of screening, positive phages were

considered as homogeneous when all plaques on a plate gave a hybridization signal. For large scale preparation of lambda phage DNA, 3×10^7 pfus of the positive phages in 0.5 ml of SM buffer were mixed with 0.5 ml $\text{MgCl}_2/\text{CaCl}_2$ (10 mM each) and 0.5 ml of the bacteria ($\text{OD}_{600} \cong 1$, *e. coli* strain LE392). After incubation at 37°C for 10 minutes, the mixture was inoculated into 400 ml NZY broth and incubated at 37°C overnight with shaking. NZY broth contains 5 g/l NaCl, 2 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/l yeast extract (LIFE Technology), 10 g/l NZ Amine (casein hydrolysate, Sigma). When cell lysis was apparent (12-18 hours), 0.8 ml of chloroform was added, and the incubation was continued at 37°C for another 15 minutes. The lysate was then centrifuged in chloroform-resistant 500-ml buckets at 7000 rpm for 10 minutes at 4°C . Supernatant was transferred to a 1 liter glass beaker and 23.4 g NaCl and 40 g polyethyleneglycol (PEG) 8000 were added. After shaking at 37°C until complete dissolving of PEG, the beaker was incubated 30 minutes on ice. Phages were then collected by spinning at 7000 rpm for 30 minutes at 4°C . Phage pellet was carefully resuspended in 6.5 ml of 1x Buffer A and transferred to a 50 ml Falcon tube. Buffer A was prepared as a 10x concentrated stock, stirred overnight at 4°C and stored at 4°C . It contains 5% (v/v) NP40, 36 mM CaCl_2 , 300 mM Tris-HCl (pH 7.5), 50 mM MgCl_2 , 1.25 M KCl, 5 mM EDTA (pH 8.0), 2.5% (w/v) Na-deoxycholate. 1x Buffer A was freshly prepared by mixing 5 ml of 10x stock buffer A, 45 ml H_2O and 20.5 μl of 14.63 M β -mercaptoethanol solution. 160 μg of DNase I (Sigma, U.S.A.) and 400 μg of RNase A were added to the phage suspension, and the mixture was incubated 30 minutes at 30°C . PEG was then removed by addition of an equal volume of chloroform, shaking and centrifugation for 10 minutes at 3000 rpm at room temperature. Phages were carefully collected from the upper layer. Conical polyallomer 10-ml tubes (Beckmann) were used to prepare a one-step gradient with a bottom layer of 750 μl of a 40% glycerol solution and an upper layer of 2.5 ml of a 5% glycerol solution. 40% glycerol solution contains 40% glycerol, 1x buffer B, 0.4 $\mu\text{l}/\text{ml}$ of 14.63 M β -mercaptoethanol. 5% glycerol solution is the same, but with a decreased amount of glycerol. 10x buffer B stock

solution contains 5% NP40, 300 mM Tris-HCl (pH 7.5), 1.25 M KCl and 5 mM EDTA. The 8 ml phage solution was finally placed on top of the two step gradient and centrifuged at 35,000 rpm for 1 hour at 4°C. Supernatant was carefully discarded and the phage pellet was resuspended in 1.6 ml of 2x ET buffer (0.04 M Tris-HCl, pH 7.5; 0.1 M EDTA) and transferred to a 5 ml cryotube (Nunc, U.S.A.). After adding 0.4 ml of a 10% SDS solution, the phage suspension was incubated overnight at 55°C. Phage DNA was then extracted with an equal volume of phenol, by gentle shaking, centrifugation at 2000 rpm for 5 minutes at room temperature, and removal of the lower organic phase. Liquid phase was further extracted with an equal volume of phenol/chloroform (1:1) in the same way. Finally, the liquid phase was extracted with an equal volume of chloroform and the upper liquid phase was saved in a 15-ml Falcon tube. DNA was precipitated with 2 volumes of 100% ethanol and the visible DNA clump was pulled out with a bent glass micropipette. After a brief washing in 70% ethanol, the DNA was dissolved in 500 µl of TE buffer. The concentration of DNA was determined by spectrophotometric absorption at 260 nm and, then, phage DNA was stored at 4°C.

4.2.2 Embryonic stem cells

E14 mouse embryonic stem cells (129ola strain; Kühn et al., 1991), were kindly provided by Dr. Klaus Pfeffer, Munich, Germany. Tissue culture plates were purchased from NUNC (Germany). Sterile plastic tubes (50 ml and 15 ml) were purchased from Falcon (Germany).

4.2.2.1 Preparation of embryonic mouse fibroblast feeder cells

In order to maintain their undifferentiated and totipotent status, embryonic stem cells (ESCs) need the presence of factors that inhibit differentiation. Early experiments showed that primary embryonic mouse fibroblast constitute a very good source of these factors (Bradley et al., 1984).

Primary embryonic fibroblast cells were obtained from mice embryos at 14-16 days post coitus (dpc). The pregnant mouse was killed and embryos were isolated from the uterus free of membranes. Embryos were transferred to a petri dish containing sterile PBS (Ca^{2+} and Mg^{2+} free, LIFE Technologies GIBCO, Germany) and heads, internal organs and limbs were removed. Carcasses were washed three times in 35 ml of sterile PBS. After the PBS washes, carcasses were quickly minced on ice with a sterile scalpel. Minced carcasses were incubated in 10 ml 0.5% trypsin/1 mM EDTA (LIFE Technologies GIBCO, Germany), supplemented with 200 $\mu\text{g/ml}$ of DNase I (Boehringer Mannheim, Germany) at 37° C for 15 minutes. A teflon mesh was sterilized for 30 minutes in boiling Ampuwa water (Fresenius, Germany). After cooling to room temperature and sequential washing in PBS and trypsin/EDTA solution, the mesh was placed into a 250 ml Erlenmeyer flask. After the trypsin incubation, the minced embryos were pressed with a sterile 5 ml syringe plunger through the mesh. A total of 50 ml trypsin/EDTA solution was used to rinse the mesh. The solution was further incubated for 30 minutes at 37° C with shaking, and then it was decanted in two 50 ml Falcon tubes. After filling the tubes with complete feeder cells medium, cells were centrifuged at 1500 rotations per minute (rpm, about 300x g) and the cell pellet was resuspended in 10 ml of complete medium. Complete feeder cell medium consists of Dulbecco's Modified Eagles Medium (DMEM, high glucose, with Na-pyruvate, LIFE Technologies GIBCO, Germany, cat. 41966052), supplemented with 10% foetal calf serum (FCS, PAA Laboratories, Germany, cat. A15-041), 2 mM glutamine (LIFE Technologies GIBCO, Germany), 100 U/ml Penicillin and 100 U/ml Streptomycin (100x Penicillin/Streptomycin solution, LIFE Technologies GIBCO, Germany). Cells were again centrifuged and the cell pellet was again resuspended in 10 ml of complete feeder cells medium. Viable cells were counted using the Trypan blue exclusion method (Sigma, Germany). Cells were plated at the density of 5×10^6 cells/dish onto 15-cm cell culture dishes containing 25 ml of complete feeder cell medium. After overnight culture at 37°C, 5% CO_2 (cell culture incubator, Haereus, Germany), the

medium was replaced with fresh medium. After 2-3 days, cells formed a confluent monolayer and were washed twice in PBS and trypsinized in 0.5% trypsin/1 mM EDTA for 5 minutes at 37° C. Cells were then centrifuged at 200 xg (about 270x g) and replated at a dilution of 1:5 and further incubated. After another 2-3 days, the cells were confluent. They were again trypsinized as above and resuspended at the concentration of about $10\text{--}20 \times 10^6$ cells/ml. Cell suspensions were pipetted into 0.5 ml aliquots into 2 ml-freezing vials (Nalgene, Germany). The content of each vial was mixed with 0.5 ml of ice-chilled 2x concentrated freezing medium. 2x freezing medium consists of 6 ml of complete feeder cells medium, supplemented with 2 ml of FCS and 2 ml of Dimethylsulfoxide (DMSO, Sigma, Germany). Cells were frozen at -80° C, and then stored in liquid nitrogen until used for the preparation of feeder cell plates.

One vial of frozen primary embryonic fibroblast cells was quickly thawed and 10 ml of complete feeder cells medium were added. After centrifugation at 200 xg (about 270x g) for 5 minutes and resuspension of the cell pellet in 10 ml of complete DMEM, cells were plated onto 5 cell culture dishes (15 cm diameter) and incubated for 4 days in cell culture incubator, or until they formed a confluent monolayer. After washing twice in PBS, cells were trypsinized at 37°C for 5 minutes. After centrifugation as above and resuspension in complete DMEM, the cells were plated at a 1:4 dilution onto 20 cell culture dishes (15 cm diameter). After 3 days, cells were treated with mitomycin C (Sigma, Germany), in order to block cell proliferation. The medium was removed and substituted with fresh medium containing 10 µg/ml of mytomicin C and the cells were put into the incubator for 3 hours. Cells were washed twice in PBS, trypsinized as above, counted and then plated at the concentration of 4×10^4 cells/cm² onto convenient plates (ranging from 96 well-plates to 10 cm-cell culture dishes).

4.2.2.2 Thawing and expanding of ESCs

An aliquot of E14 embryonic stem cells (ESCs, 129Sv strain; Kühn et al., 1991) was quickly thawed and transferred to a 50 ml Falcon tube containing 10 ml of complete ESC medium. Complete ESC medium consists DMEM, supplemented with 10% foetal calf serum (FCS, Boehringer Mannheim, Germany, cat. 210471, lot 14870302), 10^{-4} M β -mercaptoethanol (LIFE Technologies GIBCO, Germany), 2 mM glutamine, 1000 U/ml leukemia inhibitory factor (LIF, LIFE Technologies GIBCO, Germany), 100 U/ml Penicillin and 100 U/ml Streptomycin. The cells were centrifuged 5 minutes at 200 xg, the cell pellet was resuspended in ESC medium and then the cell suspension was plated onto a 6-cm cell culture dish, containing mytomicin-treated primary mouse embryonic fibroblasts (feeder plate). On the second day, the cells were washed in PBS and trypsinized in order to obtain a single cell suspension. After centrifugation, cells were resuspended in ESC medium and plated at a 1:4 dilution. After 2 days, the cells were split in the same way onto 10 cm-feeder plates at a 1:4 dilution. After another two days, cells were trypsinized and plated onto 4 gelatin pre-treated 10 cm-plates. Gelatin pre-treatment of the plates consists of an incubation with 0.1% gelatin (BDH, Germany) in Ampuwa water for few minutes, followed by two washes in PBS. After 36 hours of incubation, the cells were ready for electroporation.

4.2.2.3 Electroporation of ESCs, antibiotic selection, picking and expansion of resistant clones

60 μ g of the targeting construct were digested overnight with NotI to obtain complete linearization (clone G134.1, see below 4.3.2 "Generation of the targeting construct"). The restriction enzyme was then inactivated at 70° C for 20 minutes. The ESCs were carefully trypsinized in order to obtain a single cell suspension. After centrifugation (3 minutes at 200 xg) and resuspension in 10 ml PBS, the cells were kept in ice and counted. 7×10^6 cells were again centrifuged for 5 minutes at 200 xg. Cell pellet was resuspended in 800 μ l of cold

PBS and 20 μ l of linearized DNA (1 mg/ml) were added. The cell suspension was transferred into one electroporation cuvette (BioRad, Germany). Electroporation was obtained in a Gene Pulser electroporation apparatus (BioRad, Germany) at the following conditions: 0.24 kV, 500 μ F for 5.6 milliseconds. After electroporation, the cells were let in ice for 15 minutes and then diluted into 20 ml of complete ESC medium. The cells were then plated onto 2 gelatin-pretreated 10 cm-plates and put into the incubator. After 1 day, the medium was changed with fresh medium and after another day, the medium was substituted with selection medium, consisting of complete ESC medium supplemented with 200 μ g/ml of geneticin (G418, LIFE Technologies GIBCO, Germany). G418 is prepared as a 100x concentrated stock solution in PBS and stored at -20° C. Fresh complete ESC/G418 medium was changed every two days. After 8 days of selection, resistant clonal colonies had appeared. Single colonies were picked with the aid of plastic sterile pipette tips and plated onto single 96 well-feeder plates in complete ESC medium without G418. After one day, the clones were trypsinized with 50 μ l of Trypsin/EDTA, resuspended to single cells and, after adding 50 μ l of complete ESC medium, were replated in the same well "tryplation"). When clones had grown enough (ranging from 1 to 4 days later), they were split onto two 96 well-feeder plates and one gelatin-pretreated 96 well-plate. The expansion continued until three 96 well-feeder plates and 3 gelatin-pretreated 96 well-plates were obtained for each clone. At this point, gelatin-treated plates were used for genomic DNA extraction (see above 4.2.1.4.2 "DNA preparation from embryonic stem cells"), while cells grown on feeder plates were frozen in the following way: cells were washed once with PBS, and 50 μ l of trypsin/EDTA solution were added. Cells were incubated at 37°C for 20 minutes to obtain a single cell suspension. 50 μ l of cold complete ESC medium and 100 μ l of cold 2x concentrated freezing medium were added. 2x freezing medium consists of 6 ml of complete ESC medium with 2 ml FCS and 2 ml DMSO. After mixing the cells with the freezing medium, the plates were sealed with Parafilm (American National Can, U.S.A.) and immediately frozen at -80°C. In this way, 96 well-

plates with viable cells were stored for up to 3 months.

After Southern analysis of the DNA derived from the cells grown on gelatin-treated plates revealed the presence of positive clones, the corresponding frozen clones were thawed and expanded. Thawing of 96 well-plates was similar as described above (4.2.2.2 "Thawing and expanding of ESCs"). Cells were split progressively onto larger feeder plates (24 well-plates, 6 well-plates, 6 cm-plates, 10 cm-plates). When sufficient numbers of cells were obtained from positive clones, aliquots of cells were frozen. Briefly, the cells of one 10 cm-feeder plate were trypsinized to single cell suspension, centrifuged and resuspended in 1 ml of cold complete ESC medium. 1 ml of cold 2x freezing medium was added, and cells were frozen at -80°C in 2 ml-freezing vials (Nalgene). After a few days, frozen vials were transferred to liquid N₂, where they were stored until used for blastocyst injections. Blastocyst injection were performed according to Hogan et al. (1986).

4.2.3 *In situ* hybridization

Non-radioactive *in situ* hybridization on brain sections derived from wild type and CB1-mutant mice was performed as described in Chapter 2. The only differences were that the Tyramide Signal Amplification kit (NEN, USA) was used following the manufacturer's instructions and that diaminobenzidine was used as chromogenic substrate for visualization of the riboprobes. The riboprobe for CB1 was the same as used in Chapters 2 and 3.

4.3 Results

4.3.1 Genomic organization of the mouse CB1

4.3.1.1 Cloning of phage insert

To characterize the genomic organization of the mouse CB1 gene, a mouse genomic phage library (Kühn et al., 1991) was screened with a 1 kb *EcoRV-HindIII* fragment from CB1 ORF (CB1HIII, Fig. 4.4). From this screening, four phage genomic clones were obtained (CB1.1, CB1.2, CB1.4 and CB1.5). After phage DNA purification, restriction

mapping and Southern blot hybridization using the CB1HIII probe (Fig. 4.4), phage clones CB1.2, CB1.4 and CB1.5 appeared to have an identical 21 kb insertion; CB1.1 was about 2 kb shorter than the others, included about 1 kb more at the 5' end of the gene, and overlapped with them by about 18 kb. After Not I digestion, the insert from clone CB1.2 was subcloned into pBlueScript KS(-) (pKS). Given the length of the insert, this cloning step was quite difficult. After some fruitless attempts using standard ligation procedures, the phage insert was cloned by coprecipitation of the fragment and the plasmid in sodium acetate and 70% ethanol and resuspension in a small volume (5 μ l) of complete ligation mixture. After colony screening, using CB1HIII (Fig. 4.4) as probe, six clones were identified as positive, and after restriction mapping, three clones were shown to contain the entire phage fragment. Among these, clone G23.10 was chosen for further experiments.

4.3.1.2 Mapping of CB1 locus

Gross mapping and fragment subcloning. Several restriction digestions were carried out on clone G23.10, to reveal the gross restriction map of the CB1 genomic locus. Smaller fragments of G23.10 were subcloned to obtain more detailed information about the restriction map and to facilitate sequencing. Thus, a 4.1 kb *EcoRV-BamHI* fragment, covering about 1.5 kb of the CB1 ORF and about 2.6 kb of the 3' flanking region, was subcloned into pKS (clone G34.10; Fig. 4.4) and completely sequenced. A 3.0 kb *PstI-EcoRV* fragment, covering about 2.8 kb of the 5' flanking region of CB1 ORF and about 100 bp of the CB1 ORF, was also subcloned into pKS (clone G35.2; Fig. 4.4) and entirely sequenced. These two clones together contained the entire CB1 ORF, which was confirmed to be identical to the mouse CB1 sequence present in GenBank database (accession number U22948).

Defining limits of the targeting construct. In order to further restriction map the 5' and 3' regions of the genomic locus of CB1, two other probes were chosen, Spe6 and EIX (Fig. 4.4), and Southern blots were hybridized with these probes. This revealed the presence of two convenient restriction sites for designing the termini of the two homology arms for the

targeting construct. At a distance of about 2 kb from the 5' end of the phage insert (about 7 kb 5' to the CB1 ORF) a *EcoRV* site is present, while at a distance of about 2 kb from the 3' end of the phage insert (about 5 kb 3' to the CB1 ORF) a *HindIII* site was found (Fig. 4.4). These sites were chosen as the margins of the targeting construct.

Finding probes for screening of targeted ESCs. The fragments outside the homology margins (corresponding to the 5' and 3' end regions of clone G23.10) were subcloned (clones G88.2 and G95.1, respectively; Fig. 4.4), to find suitable restriction sites for diagnostic digestions of genomic DNA of embryonic stem cells after homologous recombination and to characterize external probes for screening these diagnostic digestions (see below, 4.3.3 "Electroporation and selection of targeted embryonic stem cell clones"). A *XbaI* and a *SpeI* site were found in clone G88.2 (5' region) and in clone G95.1 (3' region), respectively. *XbaI* is absent in the entire 5' region of CB1 locus covering the chosen homology arm and is present only at the 3' end of CB1 ORF, at a distance of about 10 kb. *SpeI* is absent in the 3' region of CB1 locus chosen as the 3' homology arm, and it is present only in the 5' region, at a distance of about 12 kb from the other *SpeI* site outside the 3' homology arm (Fig. 4.4). The regions between the *XbaI* site and the 3' end of clone G88.2, and between the *SpeI* site and the 5' end of clone G95.1, were checked for the presence of repetitive sequences by reverse Southern blot hybridization using 129Sv genomic DNA as probe. By this approach, positive hybridization signals would reveal the presence of genomic repetitive sequences. This could represent a problem for the use of a fragment as probe for genomic DNA screening. The 0.6 kb *XbaI-HindIII* fragment (XH6) in clone G88.2 and the 0.4 kb *SpeI-XbaI* fragment (SpeX4) in clone G95.1 were found to be free of repetitive sequences, as shown by lack of hybridization signal (data not shown). Therefore, these fragments were chosen as probes for the screening of targeted ESC clones (Fig. 4.4, XH6 and SpeX4).

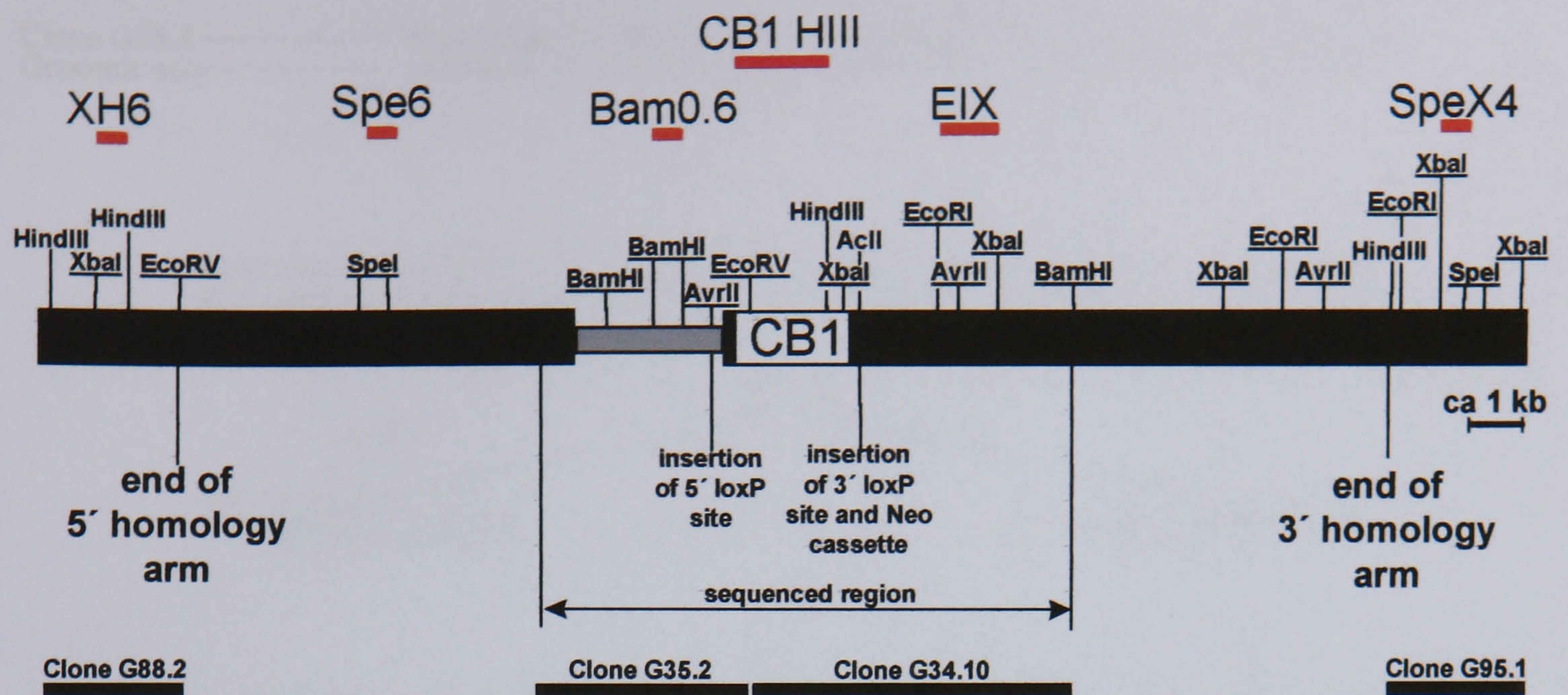


Fig. 4.4 Schematic representation of the mouse CB1 genomic locus with restriction mapping (clone G23.10). Underlined restriction sites were fully mapped. Grey box represents the intron sequence. White box represents the open reading frame (ORF) of CB1. Black boxes are the 5' and 3' flanking regions of the CB1 ORF. Red bars are fragments used as probes in Southern blot experiments. Black bars represent subclones as described in the text.

Characterization of 5' intronic sequence. Another important feature of a conditional gene targeting construct is the position where to place the loxP sites, in order to minimize the disturbance that they might cause to the normal expression of the "floxed" gene. Rat and human CB1 loci have an approximately 2 kb intron sequence at about 60 bp 5' of the CB1 ORF (Shire et al., 1995). To detect whether there was a similar intron sequence in the mouse locus, PCR was performed on mouse genomic DNA and on whole brain mouse cDNA, using the primer pairs G1-G2 and G3-G2 (Table 4.1). Primer pair G1-G2, as expected, amplified a 1.6 kb fragment both on genomic and on cDNA, whereas G3-G2 amplified a 1.7 kb fragment only on genomic DNA, indicating the putative presence of an intron sequence in the area of primer G3 (data not shown). A further analysis of the sequence of clone G35.2 (2.8 kb 5' of CB1 ORF), revealed the presence of a splice donor site (5'- GAGCAG/GTAAAT -3') at position -2102 (considering the ATG of CB1 ORF as position +1), and of a splice acceptor site (5' -GTTAG/GGTT- 3') at position -62, thus suggesting the presence of the intron sequence 5' to the CB1 ORF also in the mouse genomic locus (Fig. 4.5).

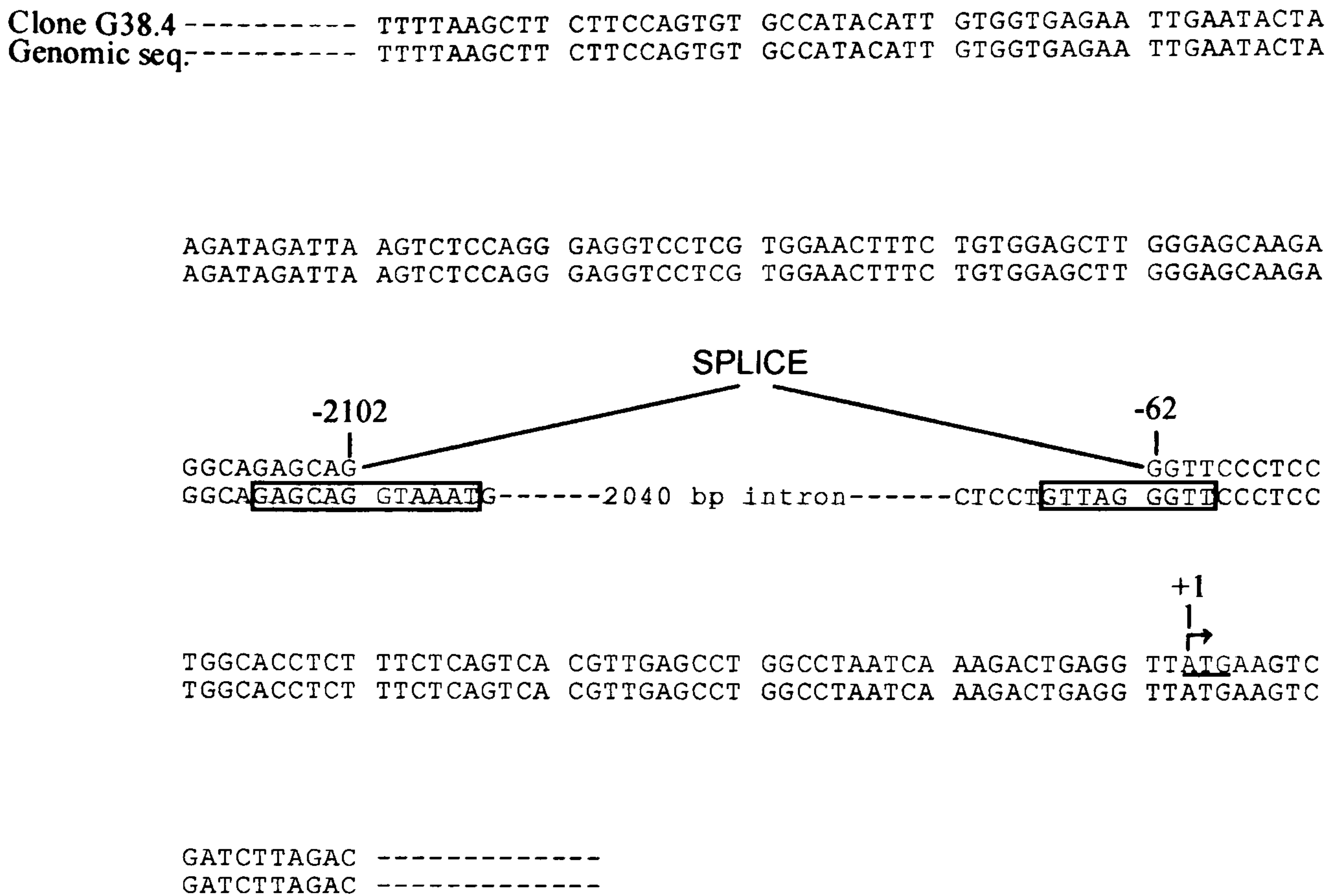


Fig. 4.5. Sequence alignment of clone G38.4 (derived from cDNA) and genomic CB1 locus (derived from clone G35.2, see Fig. 4.4), showing the presence of a 2040 bp intron from position -2102 to -62 upstream to the mouse CB1. Box, donor and acceptor splice sequences.

Primers G7 and G8 (Table 4.1) were designed to be located upstream of the putative splice donor site and downstream of the putative splice acceptor site, respectively. PCR reaction using mouse whole brain cDNA as template was performed with this primer pair and the product (about 1 kb) was cloned and sequenced (clone G38.4). By comparing the genomic sequence from clone G35.2 (Fig. 4.4) with the cDNA sequence from clone G38.4, it was possible to confirm the existence of a 2040 bp intron sequence from position -2102 to -62 upstream of the ATG of mouse CB1 (Fig. 4.5).

Determination of appropriate locations for loxP sites. At position -372, inside the intron sequence, there is an *AvrII* restriction site that is unique in the 5' region of the genomic locus. As loxP sites are rather unlikely to perturb the normal expression of a gene when they are introduced into introns (Torres & Kühn, 1997), the *AvrII* site was chosen to introduce the 5' loxP site. On the 3' side of CB1 ORF, no intron was revealed to be present, but at least two "non-canonical" polyadenylation signals are present. A unique *AcI* site, at about 2 kb downstream of the *AvrII* site, is present between the first and the second polyadenylation

signal. This was considered to be a good position to place the 3' loxP site.

In conclusion, restriction mapping, Southern hybridization and sequencing of the CB1 genomic locus revealed the presence of a single ORF encoding for CB1, confirmed the presence of a 2 kb intron sequence at about 60 bp 5' to CB1 ORF and allowed me to design a conditional gene targeting construct.

4.3.2 Generation of the targeting construct

Given the sequence information and the restriction mapping data, the construction of the gene targeting construct was carried out. Fig. 4.6 shows the cloning steps that were necessary to achieve this construct. Restriction sites were introduced, during the cloning steps, in order to facilitate the Southern blotting screening of targeted ESC clones. Major difficulties were encountered at the last steps, because of the dimensions of the inserts and vectors. In some cases, i.e. steps 8-10, bacterial colony lifts were necessary to find positive clones, and, given the low yields of large plasmids, high amounts of bacterial cultures (up to 3 liters) were used for plasmid preparations. No blunt ligation was used in the steps where big fragments (> 5 kb) were to be cloned. An *Xba*I site was left in the neighboring 3' region of the 5' loxP site (Fig. 4.6, step 2), while a *Spe*I site was carried on from the subcloning of the FRT-PGK-Neo cassette in position 3' to the second FRT site (Fig. 4.6, steps 5, 6 and 8). At step 2, a *Not*I site carried from the loxP original plasmid (pFTM-loxP), was destroyed by digestion, blunting and self-ligation (clone G105.16 to clone G109.2), in order to maintain a unique *Not*I site in the 5' polylinker region of the final construct, since a single restriction site, external to the targeting construct, is necessary for linearization of the plasmid prior to electroporation of ESCs.

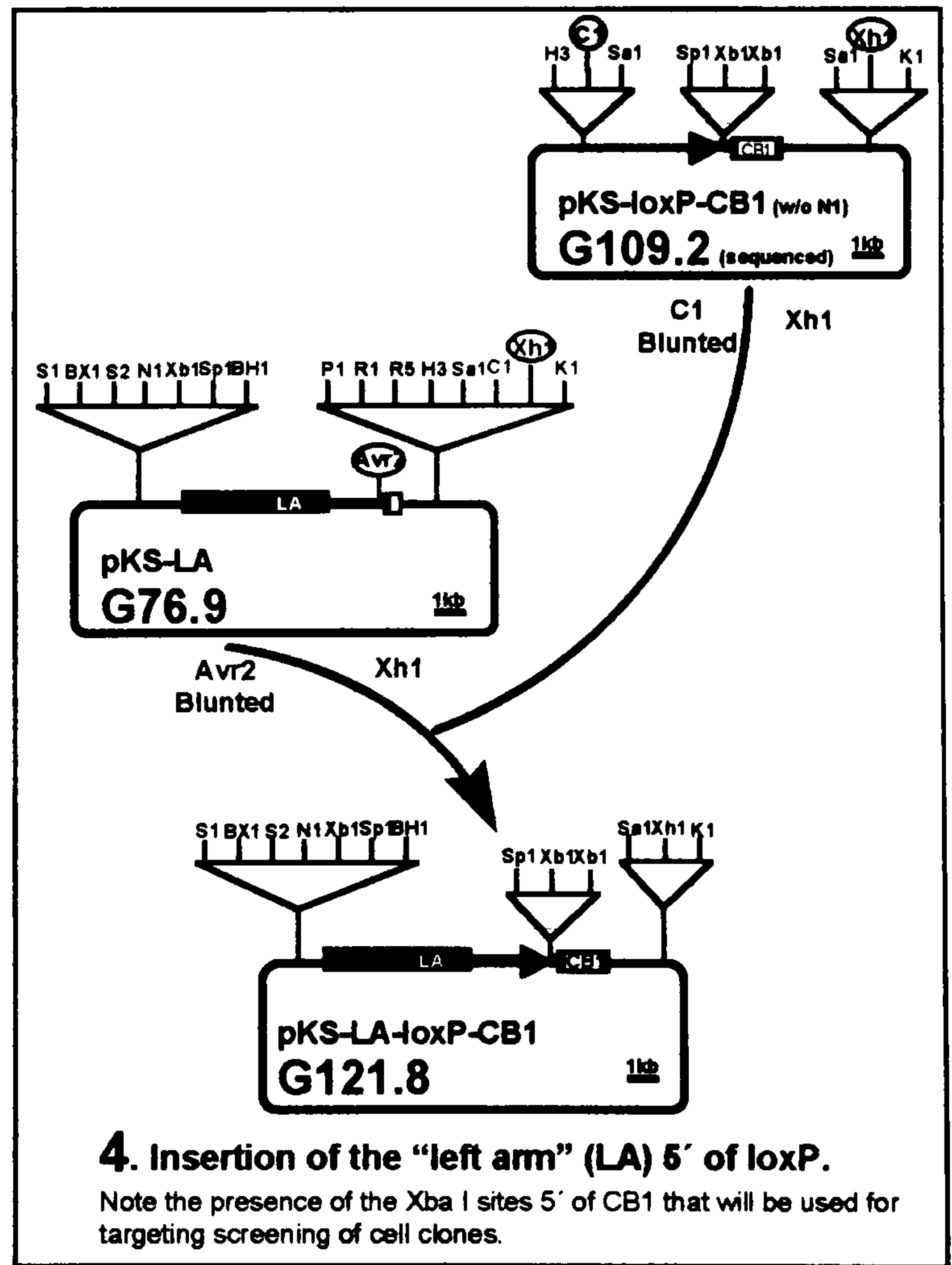
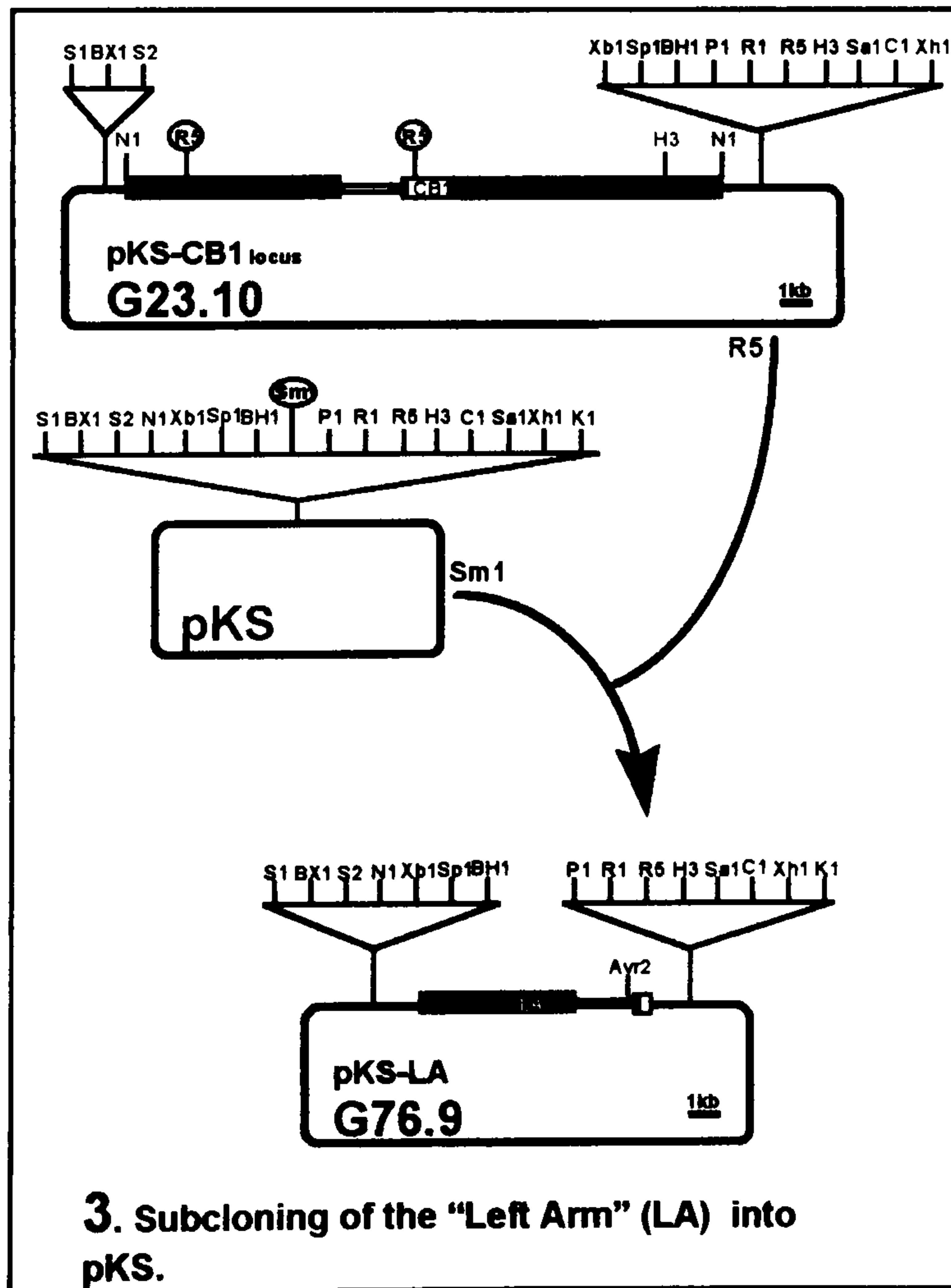
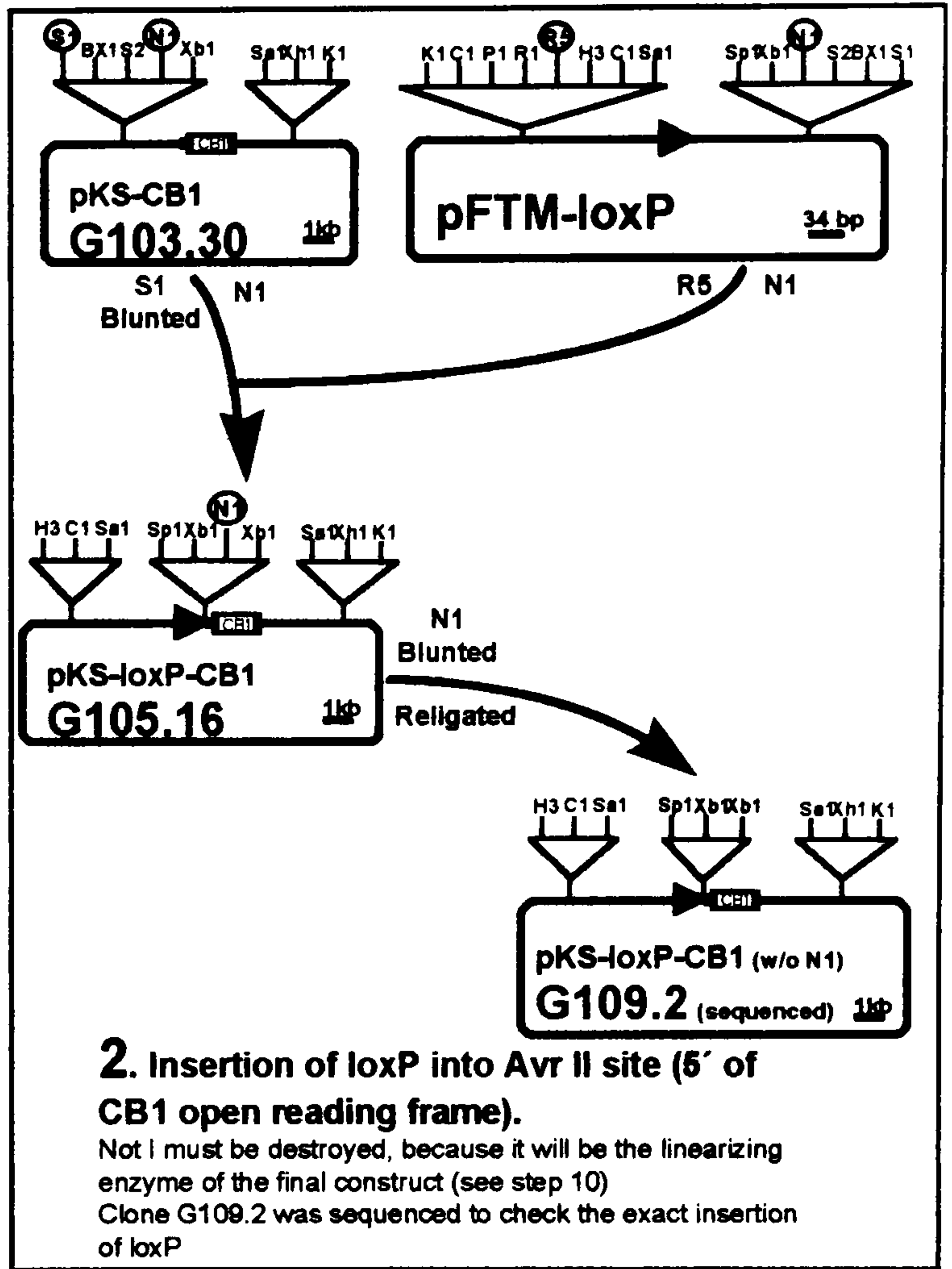
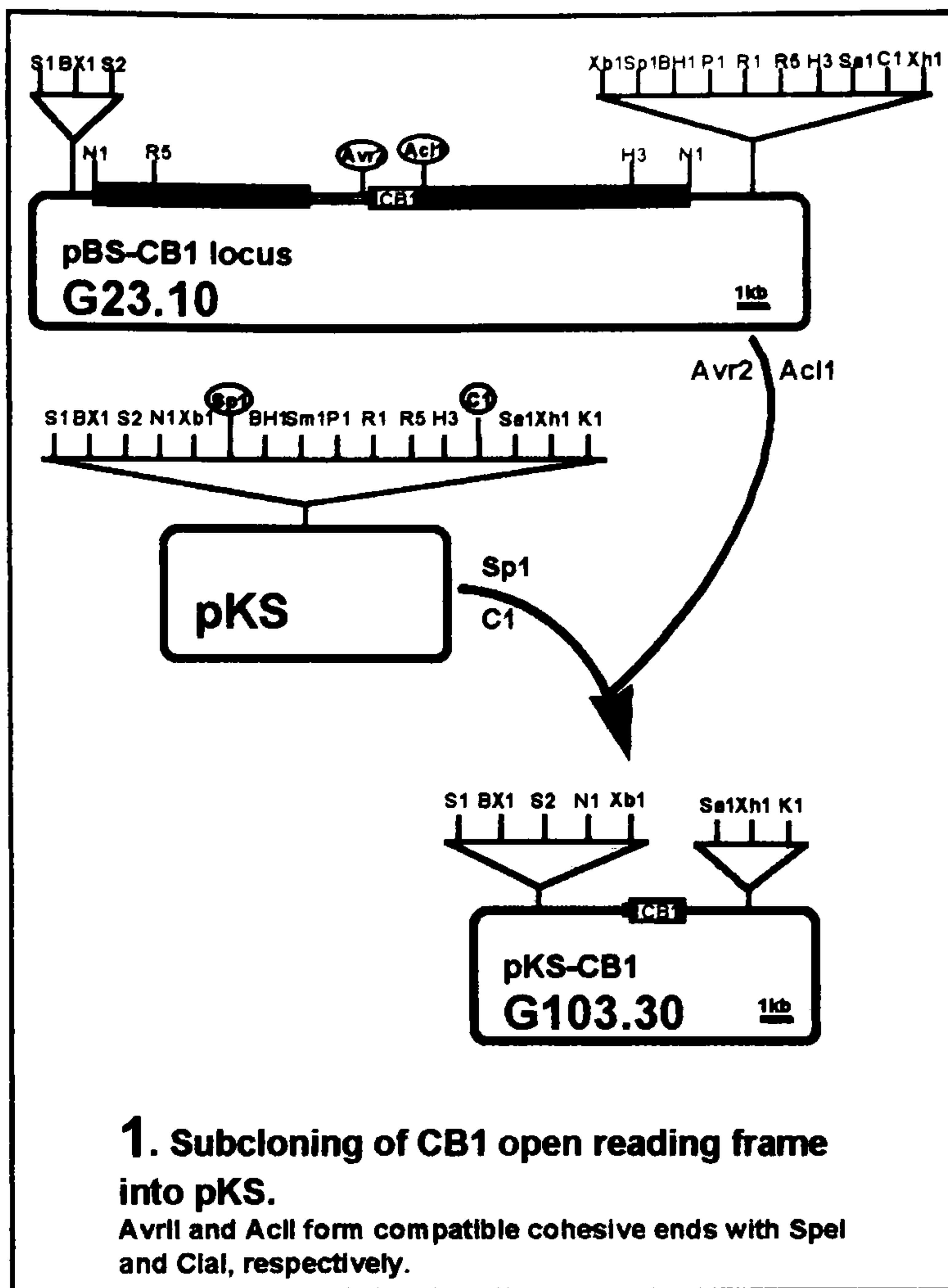


Fig. 4.6 (continued)

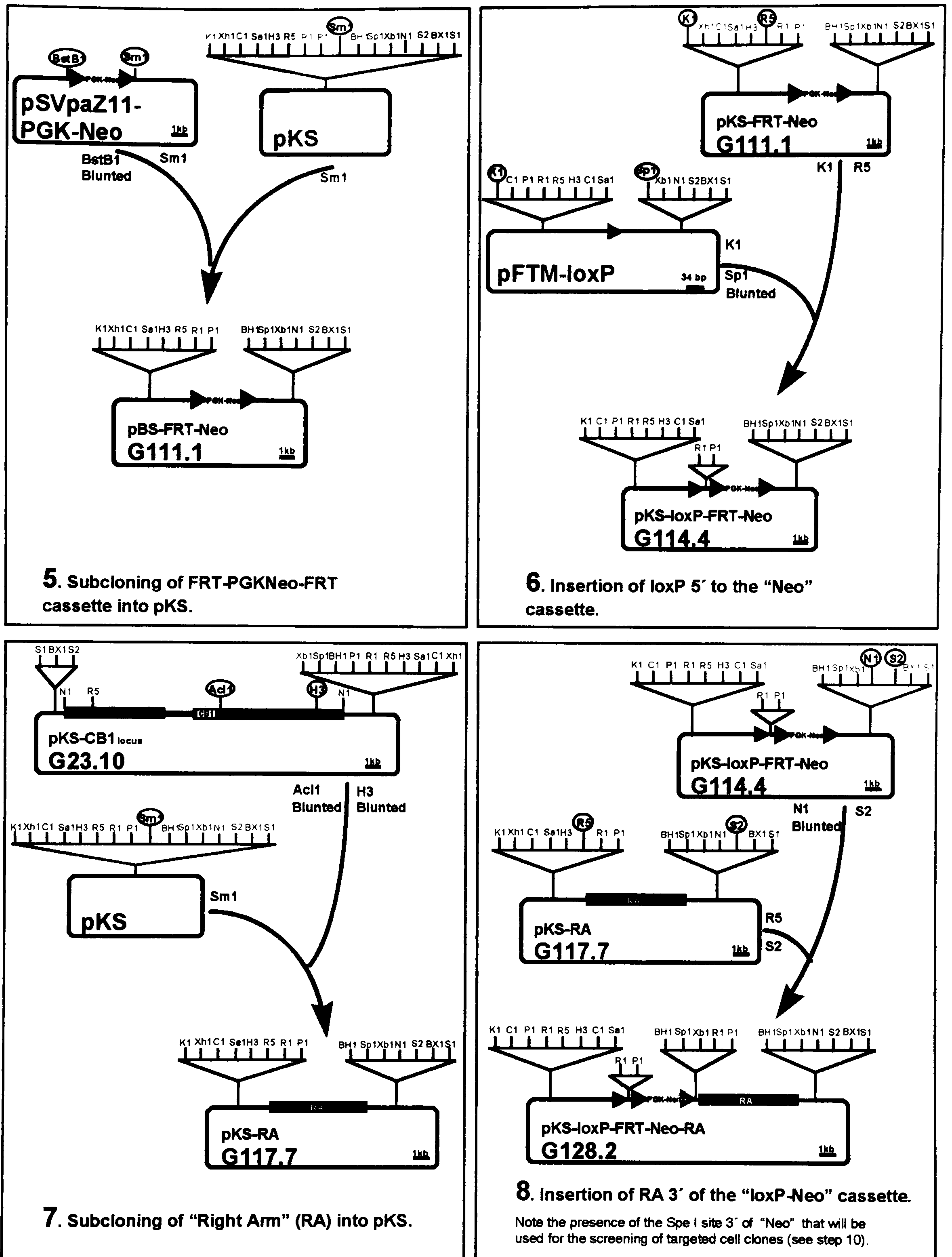


Fig 4.6 (continued)

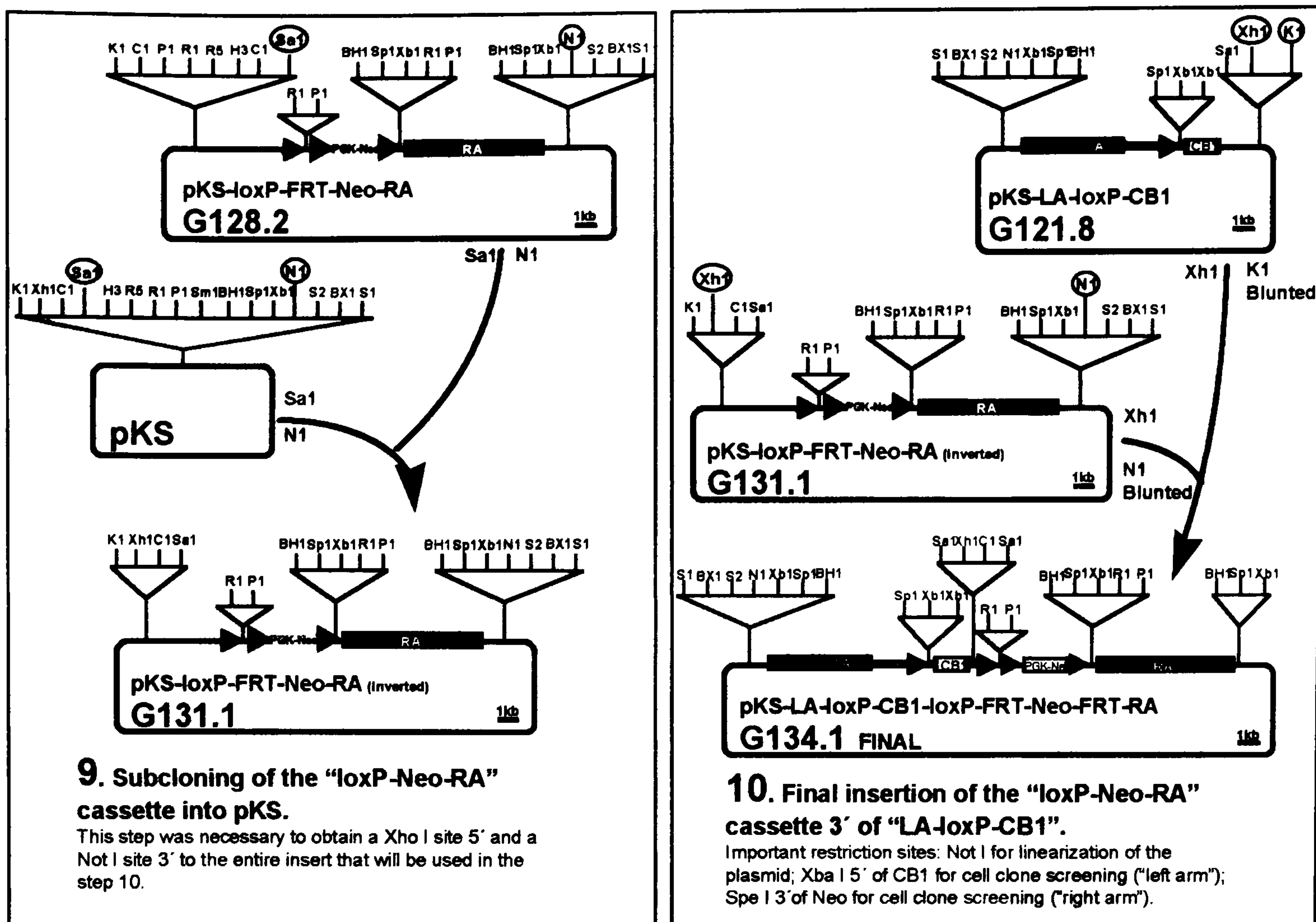


Fig. 4.6 (see also previous two pages). Schematic representation of the cloning steps performed to obtain the gene targeting construct. Steps 1 to 4 were performed to assemble the left arm of homology (LA, black box), the 5' loxP site (loxP, red triangle) and the CB1 ORF (CB1, white box) (clone G121.8). Steps 5 to 8 were necessary to assemble the 3' loxP site (loxP, red triangle), the FRT-PGK-Neo cassette (FRT, black triangle; PGK-Neo, yellow box) and the right arm of homology (RA, black box) (clone G128.2). Steps 9 and 10 were used to assemble the two arms of the construct, in order to obtain the final gene targeting construct G134.1. Grey box represents the intron sequence. Scale bars are 1 kb, but the backbone plasmid sequence, the loxP site, the FRT site and the polylinker regions are not represented in correct proportion. At each step, used restriction enzymes are bold and circled. Abbreviations: pKS, pBlueScript KS(-); restriction enzymes: *Acl*1, *Acl*I; *Avr*2, *Avr*II; *BH*1, *Bam*HI; *BX*1, *Bst*XI; *C*1, *Cl*aI; *H*3, *Hind*III; *K*1, *Kpn*I; *N*1, *Not*I; *P*1, *Pst*I; *R*1, *Eco*RI; *R*5, *Eco*RV; *S*1, *Sac*I; *S*2, *Sac*II; *S*a1, *Sal*I; *S*m1, *Sma*I; *S*p1, *Spe*I; *X*b1, *Xba*I; *X*h1, *Xho*I.

Before proceeding to embryonic stem cells electroporation, the final clone G134.1, was sent for sequencing of the complete CB1 ORF and of the most important regions of the construct (lox P sites, FRT sites, junctions between different fragments). Sequencing data confirmed the identity of the construct and the lack of mutations.

4.3.3 Electroporation and selection of targeted embryonic stem cell clones

60 μ g of clone G134.1 were linearized by *NotI* digestion and 20 μ g were used for electroporation of E14 mouse embryonic stem cells (129Sv strain; Kühn et al., 1991). After 8 days of selection in geneticin (G418) at 200 μ g/ml, approximately 400 resistant clones were picked, grown in 96-well plates and subsequently expanded for screening and freezing. In a loxP gene targeting construct, there are three regions of homology where homologous recombination can occur: the region 5' to the first loxP site (left arm of homology, LA), the sequence of interest (in this case: the CB1 ORF) and the region 3' to the second loxP site (right arm of homology, RA). To obtain the desired gene targeting, recombination events should occur exclusively in the first (LA) and the third (RA) of these homology regions. If one of the homologous recombinations between gene targeting construct and genomic DNA involves the second of these homology regions (CB1 ORF), one of the two loxP would be inevitably lost, thus blocking the Cre/loxP approach. In the 3' region (RA) of clone G134.1, the selection cassette PGK-Neo is present, conferring the resistance to the drug G418 in the ESCs. It is therefore very likely that G418-resistant ESC clones contain the RA of homology. Thus, the primary screening was addressed to the LA, using *XbaI* genomic DNA digestions of picked and expanded clones. Southern blot hybridization, using the probe XH6 (Fig. 4.4) revealed the presence of two bands at about 10 kb (wild type allele) and at 8.5 kb (targeted allele) in 11 clones (about 3% recombination efficiency) (Fig. 4.7A, B). As expected, a further digestion with *SpeI* and subsequent Southern blot hybridization using the probe SpeX4, revealed that the wild type (WT) band at about 12 kb and the correctly recombined band at about 6.5 kb were also present in all the 11 clones (Fig. 4.7A, C). Additional random integrations of the Neo cassette were checked by restriction digestion of genomic DNA of positive ESC clones with three different enzymes (*XhoI*, *SalI* and *SpeI*) and Southern hybridization using a 600 bp-long *XbaI*-*PstI* fragment of the Neo gene as a probe. Only clones that showed a single positive band with all three digestions (not shown), indicating a

single correct targeted site of integration, were used for further experiments. Two clones out of the eleven targeted ones showed additional random integration sites (data not shown).

4.3.4 Blastocyst injection, generation of chimeras and germ line transmission

Three ESC clones (clones SD7, SSB12 and MBH2) were chosen for injection of C57BL/6-derived blastocysts. Table 4.2 summarizes the results of blastocyst injection experiments. A total of 538 blastocysts were injected with either of the three ESC clones. Of the 77 pups born from these experiments, 29 were chimeric (37,7%), with various values of chimerism.

Clone	Number of injected embryos	Number of born pups	Number of chimeras (% of chimerism)	Germ line transmission
SD7	152	17	7 (20-80%)	YES
SSB12	197	42	14 (3-100%)	YES
MBH2	189	18	8 (2-80%)	YES

Table 4.2. Summary of blastocyst injection experiments. The percentage of chimerism was empirically evaluated based on the coat colour of chimeras.

Chimeras derived from all the three ESC clones were mated with wild type C57BL/6 mice to check the germ line transmission of the targeted allele. Germ line transmission was revealed by the coat colour of offspring (black, not germ line; agouti, germ line transmission of agouti ESCs), and was confirmed by PCR (Fig. 4.7D) and by Southern blot experiments (data not shown) of tail-derived genomic DNA. Two primers, G50 and G51, were used for PCR analysis (Tab. 4.2). Both primers correspond to wild type sequences, one located 5' (G50) and the other located 3' (G51) of the AvrII restriction site, where the 5' loxP site was inserted.

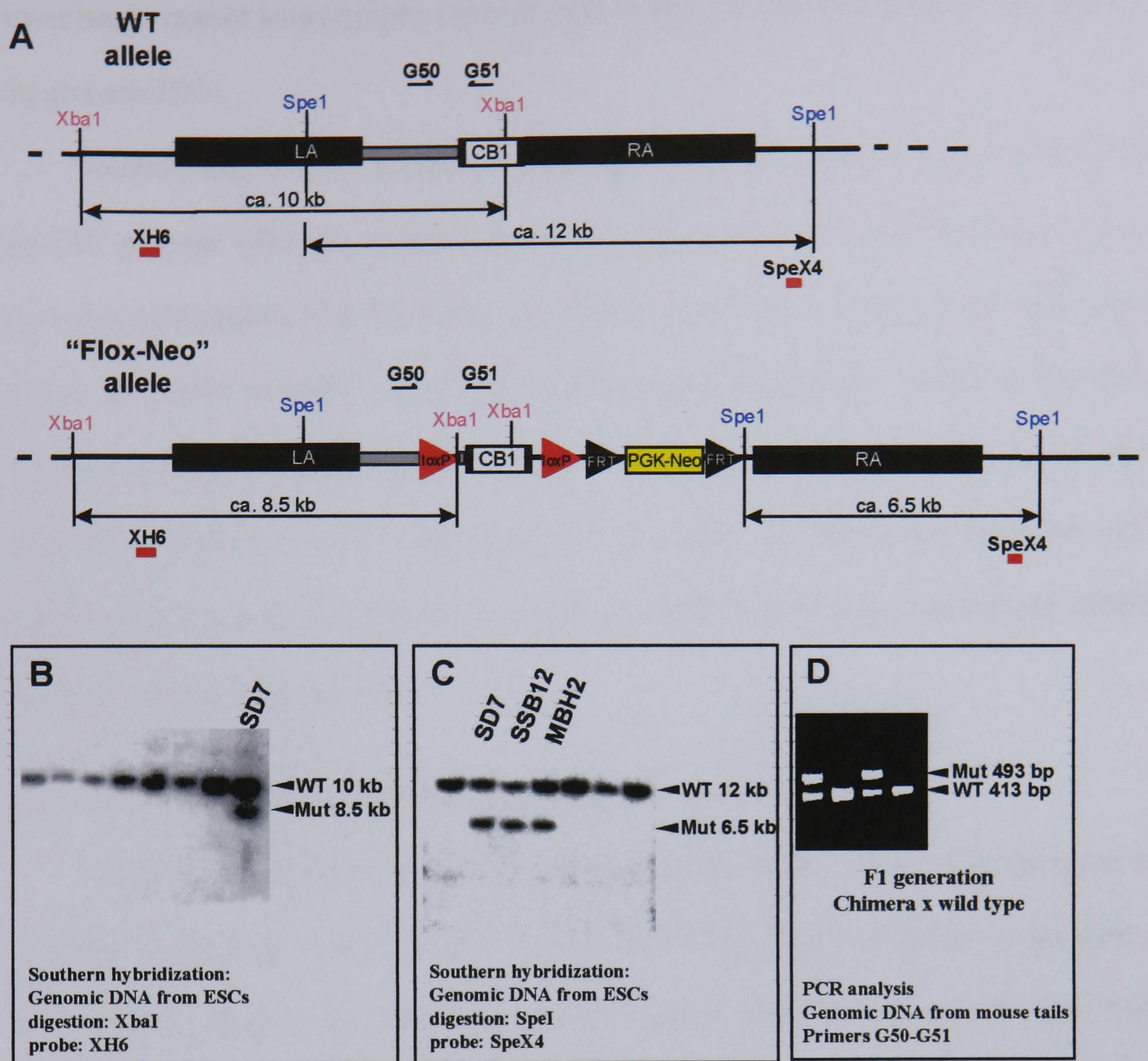


Fig. 4.7. Analysis of targeted ESC clones and germ line transmission of the gene targeting construct. A. Schematic representation of the wild type (WT) and "flox-Neo" alleles. B. Southern blot analysis of G418-resistant ESC clones. Clone SD7 (and also SSB12 and MBH2, not shown) contains the correct recombination of the 5' region of the targeting construct (primary screening, left arm of homology, LA). C. Southern blot analysis of G418-resistant ESC clones. Clones SD7, SSB12 and MBH2 contain the correct recombination of the 3' region of the targeting construct (secondary screening, right arm of homology, RA). D. PCR analysis of germ line transmission. Agouti offsprings derived from crossing of wild type C57BL/6 mice with clone SD7-derived chimeras. Double PCR bands correspond to germ line transmitted animals. Black bars, homology arms (LA and RA); grey bar, intron sequence; white bar, CB1 open reading frame; yellow bar, PGK-Neo cassette; red triangle, loxP site; black triangle, FRT site; G50 and G51, primers used for PCR analysis; XH6 and SpeX4, probes used for Southern blot analysis.

Using these primers, wild type alleles give an amplicon of 413 bp, whereas targeted alleles give a 493 bp amplicon (Fig. 4.7A, D). These two bands are clearly distinguishable in a 1.5 % agarose gel, and easily allow to distinguish between wild type homozygosity (only the

lower band), mutant homozygosity (only the higher band) and heterozygosity (both bands) in the genomic DNA.

Southern blot analysis was performed on mouse tail DNA with the same conditions as for ESC genomic DNA and gave identical results (not shown). All three lines gave rise to germ line transmission, with efficiencies (evaluated as percentage of agouti pups per litter), ranging from 10% to 100%. These germ line-transmitted animals were defined as the "flox-Neo" line, as they contain the complete sequence of the targeting construct, with the "floxed" CB1 and the PGK-Neo cassette flanked by FRT sequences. The following experiments were carried out using only mice derived from SD7 and SSB12 cell clones, keeping the MBH2 derived mice as a "back-up" line.

4.3.5 Generation of CB1 "Null mutant" ("CBN" mouse line)

To check the efficiency of the Cre-mediated excision of the "floxed" CB1 gene and to generate a CB1-null mutant (CBN) mouse line to be used for further experiments, heterozygous animals of the "flox-Neo" line were crossed with "Cre-deleter" mice. The "Cre-deleter" mice are a transgenic line that express Cre recombinase under the control of a human cytomegalovirus minimal promoter (P_{Bi-2} , Baron et al., 1995). Given the relatively low specificity of the promoter, these mice express rather high levels of Cre recombinase in virtually all the cells of the body, including germ cells (Schwenk et al., 1995). The excision of CB1 (CBN allele) was checked by PCR and Southern blotting of tail-derived DNA from mice of the F1 generation (offspring of heterozygous "flox-Neo" mice and heterozygous "Cre-deleter" mice). PCR was performed using three primers in the same reaction: G50, G51 and G54. G50 and G51 are described in the previous paragraph (Fig. 4.7A, D), while G54 is a reverse primer located in the 5' end region of the "PGK-Neo" cassette (Table 4.2 and Fig. 4.8A). Best results were obtained when the final concentrations of the primers were as follows: G50 1 nmol/ml, G51 0.5 nmol/ml, G54 0.7 nmol/ml. On wild type alleles, which lack the "PGK-Neo" cassette, the G50/G51 primer pair gave an amplification product of 413 bp

(Fig. 4.8C). On CB1-deleted alleles (CBN), G51 does not have the template to anneal with, while the PGK-Neo cassette is moved closer to the G50 primer (Fig. 4.8A). In this case, the G50/G54 primer pair gave a 342 bp amplification product, clearly distinguishable from the wild type G50/G51 product on a 1.5% agarose gel (Fig. 4.8C). Also in this case, it was easy to discriminate between homozygosity or heterozygosity.

Southern blot analysis was performed by digesting tail-derived genomic DNA with the restriction enzyme *SpeI*. A 0.6 kb *BamHI* fragment in the LA of homology, located 3' to the *SpeI* site in the same area, was used as a probe (Fig. 4.4 and Fig. 4.8A, B). Wild type alleles gave an approximately 12 kb band that was the same as described in the previous paragraph for the *SpeX4* probe (Fig. 4.7C). An *SpeI* site in the 3' flanking area of the first loxP site (i.e. inside the region excised by Cre recombinase) was kept during the cloning steps of the targeting construct (Fig. 4.6, step 2). Moreover, another *SpeI* site is present in the 5' region inside the PGK-Neo cassette (Fig. 4.8A). Therefore, in case of Cre-mediated deletion of CB1 ("CBN allele"), Southern blot hybridization gives a 3.3 kb band, whereas a "flox-Neo" allele (i.e., where no recombination had occurred), gives a 2.7 kb band (Fig. 4.8B). Southern blotting analysis gave the same conclusions as the PCR experiments described above.

Mice carrying a CBN allele were then crossed with wild type C57BL/6 mice to check the germ-line transmission of the mutation. The efficiency of Cre-mediated deletion of CB1 ORF was very high. In the F1 generation (offspring of heterozygous "flox-Neo" mice and heterozygous "Cre-deleter" mice), 50% of the mice containing the Cre transgene carried the CBN locus, thus showing the high activity of Cre-mediated CB1 excision *in vivo* (Fig. 4.8B, C). Nevertheless, in the F1 generation, the Cre-mediated deletion might have a "mosaic" character, with only a limited number of cells in which the recombination has actually occurred. However, also in F2 generation (F1 crossed with wild type C57BL/6 mice), about 50% of the offspring carried the deletion of the CB1 ORF band (Fig. 4.8D). This gives a strong indication of the high efficiency of the Cre-mediated excision of "floxed" CB1 *in vivo*.

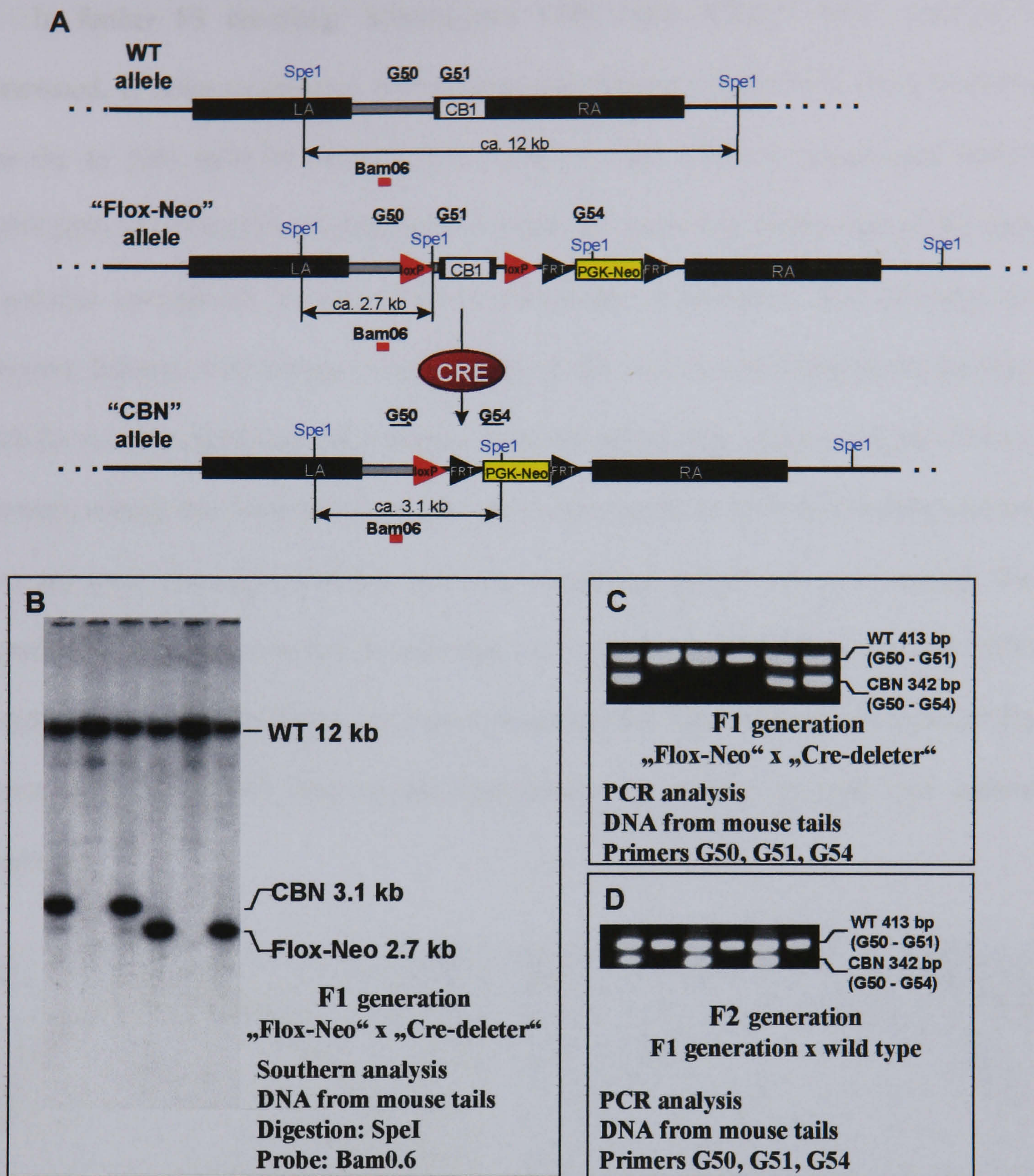


Fig. 4.8. Cre recombinase-mediated deletion of CB1 ORF and generation of the "CBN" (CB1-null) mouse line. A. Schematic representation of the wild type (WT) allele, of the "flox-Neo" allele and of the "CBN" allele after the Cre-mediated deletion of the CB1 ORF. Symbols are as in Fig. 4.7. B. Southern blot analysis of individuals of the F1 generation obtained by mating "flox-Neo" with "Cre-deleter" mice. About 50% of the offspring mice containing the Cre transgene carried the deleted allele. C. PCR analysis of F1 generation as in B, but not in the same order. D. PCR analysis of mice of the F2 generation obtained by mating "F1" deleted mice (as in A and B) with C57Bl/6 wild type mice. 50% of the offspring carry the germ line-transmitted deletion, indicating a very efficient Cre-mediated deletion of the CB1 locus already at F1 generation.

In further F3 breedings, heterozygous CBN mice (CBN/+) were successively intercrossed, in order to generate homozygous null mutants (CBN/CBN). Such breedings gave rise to 53% (63/119) heterozygous (CBN/+), 24% (29/119) homozygous mutant (CBN/CBN) and 23% (27/119) wild type (+/+) mice, as counted in the first days of life. Such proportions corresponds to the expected Mendelian transmission, thus revealing no embryonic lethality of the mutation. The majority of mice were sacrificed during the first days of life for *in vitro* experiments using primary neuronal cultures (see Chapter 5 of this Thesis). However, animals that were allowed to age (up to four months, at the time of writing) did not show any gross phenotypical defect, and body weight and growth rate were normal. The expression of CB1 was checked in wild type (+/+) and homozygous mutant (CBN/CBN) littermates by *in situ* hybridization on coronal brain sections. Fig. 4.9 shows the lack of CB1 expression in CBN/CBN mice in the hippocampus, in contrast to wild type control littermates.

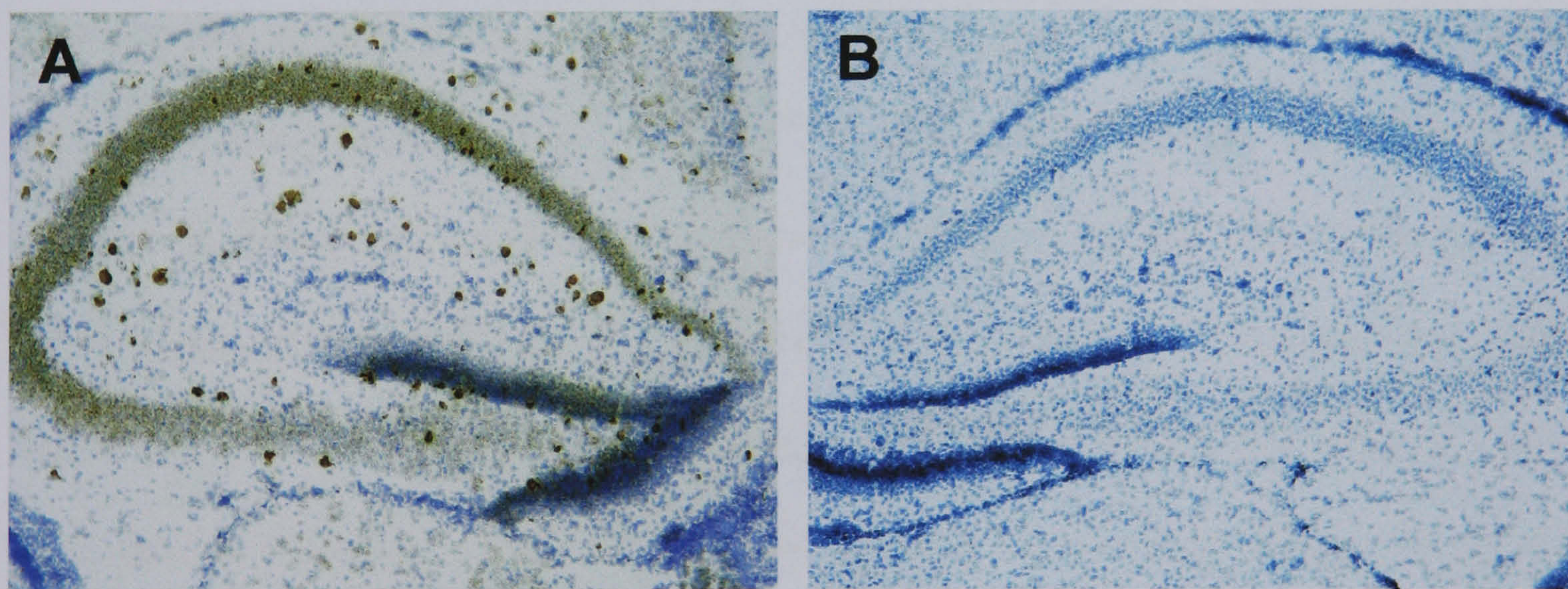


Fig. 4.9. Non-radioactive *in situ* hybridization on coronal sections of hippocampi derived from wild type (A) and CBN/CBN (B) mice. The CB1 riboprobe was the same as described in Chapter 2. Diaminobenzidin (DAB) was used as chromogenic substrate. Both sections were counterstained with toluidine blue.

In conclusion, the Cre-mediated excision of CB1 was shown to occur in a reliable and efficient way. This is an important observation, because the efficiency of the Cre/loxP system in the mouse can be influenced by many events that are very often unpredictable (Torres &

Kühn, 1997). Therefore, these results constitute a strong indication that the "conditional" deletion of CB1 will be possible by using "floxed CB1" mice as described in the next paragraph and suitable Cre-expressing mouse lines. Moreover, the lack of expression of CB1 in CBN/CBN mice will constitute a valuable tool for *in vitro* investigations (e.g. see Chapter 5).

4.3.6 Generation of "floxed mutant" "floxed CB1" line)

As mentioned in the Introduction of this Chapter, one of the key points of a conditional gene targeting approach is the completely wild type expression of the gene of interest before the Cre-mediated excision (i.e. before the crossing with a Cre-expressing mouse line). To achieve this result, the length of sequences artificially introduced into the genomic locus must be as short as possible. Any long sequence introduced might cause alterations in the wild type expression of the gene of interest. For this reason, given the presence of the 1.8 kb "PGK-Neo" cassette in the 3' region of CB1 locus, the "flox-Neo" mouse line obtained from the gene targeting and the blastocyst injection of the ESCs, cannot yet be considered as optimal for a further conditional gene mutation. The "PGK-Neo" cassette used for the gene targeting construct is flanked by two recombination sites, different from loxP (Fig. 4.2B and Fig. 4.7A). These are FRT sites, and they are recognized by a yeast derived recombinase, called flipase (flp) (Dymecki, 1996). The flp/FRT system is less efficient than the Cre/loxP one at 37°C, but still maintains a certain activity (Buchholz et al., 1996). Since efficient transfection conditions of a flipase-expressing vector into ESCs are still to be established, it was decided to induce the excision of the FRT-flanked PGK-Neo cassette by crossing the "flox-Neo" mouse line with a "flipase-deleter" mouse line. In this transgenic mouse line, the recombinase is under the control of the constitutively active promoter of the human β -actin gene and is, therefore, rather ubiquitously expressed (Dymecki, 1996). The deletion of the "PGK-Neo" cassette in the progeny of this cross was checked by PCR and by Southern blot analysis on tail-derived genomic DNA. PCR was performed using the primers G52 and G53 (Tab. 4.1

and Fig. 4.10A). These primers anneal to wild type sequences on either side of the *AcII* restriction site where the loxP-PGK-Neo cassette was inserted (Fig. 4.6). On wild type alleles, G52/G53 gave an amplification product of 418 bp, whereas in the "PGK-Neo"-deleted alleles, the product is 520 bp, clearly distinguishable from wild type in a 1.5% agarose gel. Alleles where no deletion has occurred should give a >2 kb band. However, in a heterozygous state, the presence of the much shorter wild type and "Neo-deleted" products, which are more efficiently amplified in the PCR reactions than the >2 kb band, made it impossible to distinguish between animals where the recombination was complete and animals where a "mosaic" situation was present. In fact, given the relatively low efficiency of the flp/FRT system at 37°C (Buchholz et al., 1996), it was expected that in the F1 generation ("flox-Neo" heterozygous mice crossed with heterozygous "Flp-deleters"), single animals would be mosaic, with the deletion of the "PGK-Neo" cassette only in limited number of cells. However, in the F1 generation, about 50% of the animals carrying the Flp transgene showed to have at least a mosaic deletion of the "PGK-Neo" cassette (Fig. 4.10B). The "mosaic" situation of the F1 generation was confirmed by checking the germ line transmission of the deletion of the "PGK-Neo" cassette. Only mice where the deletion had occurred in germ line cells were expected to be able to transmit the mutation to the next generation, after mating with wild type C57BL/6 mice (F2 generation). Indeed, by using PCR analysis (using primers G52 and G53) on tail-derived DNA from offspring of the F2 generation, only about 10% of the mice were shown to contain the deletion of the "PGK-Neo" cassette (Fig. 4.10C). These mice were further used for breeding a new mouse line, called "Floxed CB1" line. To confirm the reliability of the PCR-based genotyping, Southern blot analysis was also conducted in F3 generation mice (F2 generation "Floxed CB1" crossed with wild type C57BL/6 mice) (Fig. 4.10D). *Bam*HI digests of genomic DNA from mouse tails were electrophoresed, blotted and hybridized with the CB1 HIII probe (Fig. 4.4).

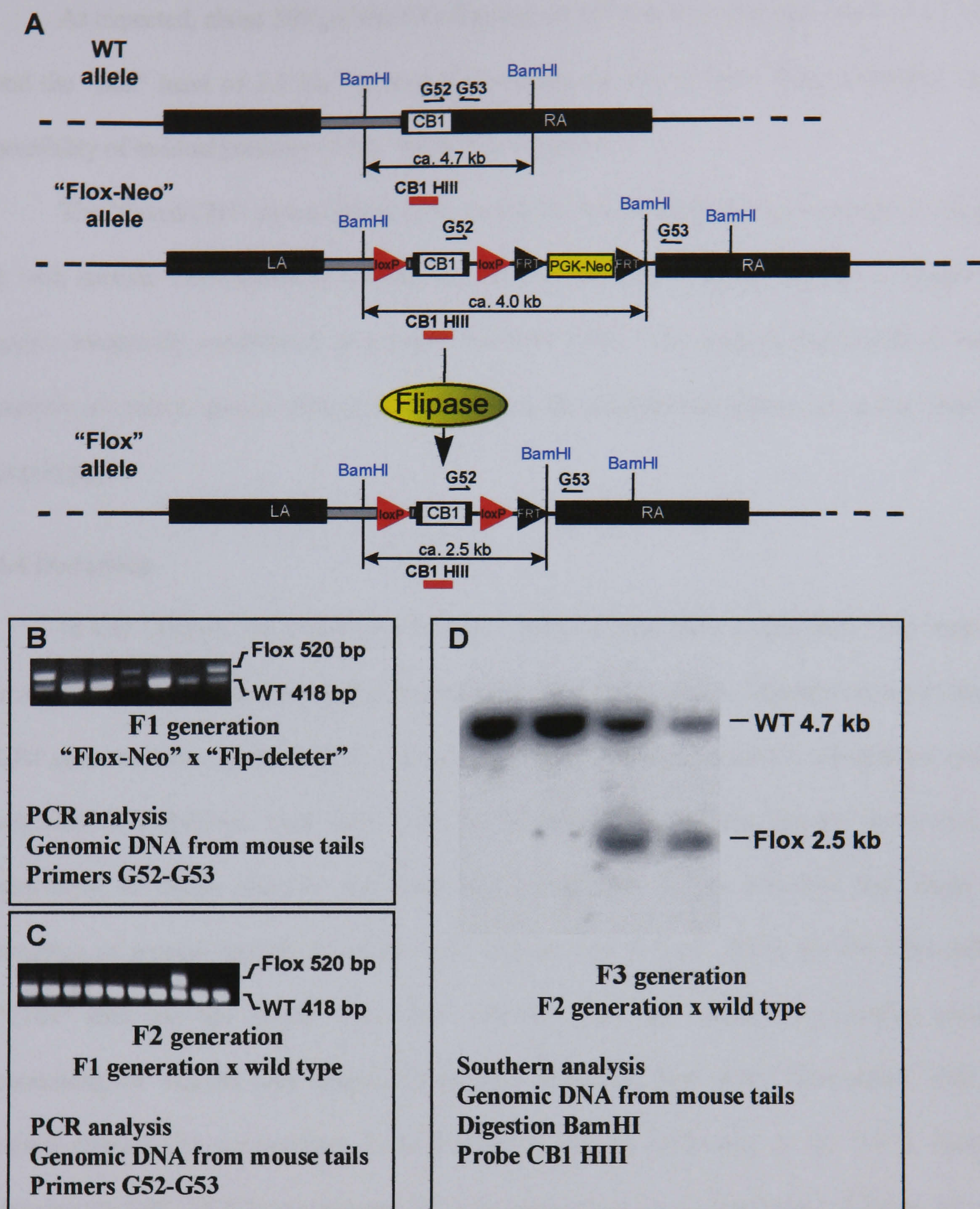


Fig. 4.10. Flipase-mediated deletion of the "Neo" cassette and generation of the "floxed CB1" mouse line. A. Schematic representation of the wild type allele (WT), the "flox-Neo" allele and the "flox" allele after the flipase-mediated deletion of the "Neo" cassette. Symbols are as in Fig. 4.7. B. PCR analysis of individuals of the F1 generation obtained by mating heterozygous "flox-Neo" with heterozygous "Flp-deleter" mice. About 50% of the offspring mice containing the Flp transgene carried the deleted allele. C. PCR analysis of F2 mice obtained by mating F1 deleted mice (as in A) with C57BL/6 wild type mice. Only 10% of these mice carried the germ line-transmitted deletion, indicating a "mosaic" status of most mice that carried this deletion in the F1 generation. D. Southern blot analysis of 4 individuals of the F3 generation (F2 x wild type).

As expected, about 50% of the F3 offspring carried both the wild type band of 4.7 kb and the "flox" band of 2.5 kb. No animal contained the 4.0 kb band, thus, excluding the possibility of residual presence of the "PGK-Neo" cassette.

The "floxed CB1" mouse line was then ready for further experiments, in which crossing it with specific Cre-expressing mouse lines should generate progeny carrying a spatially and/or temporally conditioned deletion of the CB1 ORF. This progeny should allow the analysis of region-specific functions of CB1 and the cannabinoid system in mouse brain physiology.

4.4 Discussion

In this Chapter, the generation of CB1 mutant mouse lines is described. The steps involved in this task were: screening of a genomic DNA phage library, characterization of the CB1 genomic locus, generation of a "conditional" gene targeting construct, transfection and selection of embryonic stem cells, injection of positive clones into mouse blastocysts, generation of mouse chimeras and germ line transmission of the mutation, and, finally, breeding of mutant animals to obtain two different mouse lines. These are the CB1-null ("CBN" line) and the "floxed CB1" line. The first one was obtained by crossing mice containing the original gene targeted sequences "flox-Neo" line) with "Cre-deleter" mice, which express Cre recombinase in an ubiquitous manner (Schwenk et al., 1995), thus, deleting the CB1 ORF in the progeny. F1 mice were crossed with wild type C57BL/6 mice, obtaining F2 progeny to check the germ line transmission of the deletion of the CB1 ORF. In this way, two aims were accomplished: first, the Cre recombinase was actually able to delete the "floxed" CB1, *in vivo*, thus, providing the direct demonstration that the "conditional" gene targeting approach indeed works in the CB1 locus; second, CB1 null mutant mice ("CBN" line) were generated in order to analyze the CB1 functions in experimental conditions where no spatio-temporal resolution is required, such as in *in vitro* cell biology assays (see Chapter 5). The second mouse line generated during the work was obtained by crossing the gene-

targeted allele with "flipase-deleter" mice, expressing the enzyme flipase in an ubiquitous manner (Dymecky, 1996). F1 mice were then used for further crossing with wild type C57BL/6 mice to check for the germ line transmission of the flipase-mediated deletion of the "PGK-Neo" cassette. Flipase is able to recognize and recombine short sequences, called FRT, in an analogous fashion as Cre recombinase with loxP sites (Buchholz et al., 1996). In the gene targeting construct, FRT sites, flanked the selection cassette "PGK-Neo", necessary for the selection of positive targeted clones in ESCs. As the presence of this cassette might be deleterious for a perfect wild type expression of the "floxed" gene of interest, the deletion of the selection cassette is a necessary step for the generation of an optimal "conditional" inactivation of any gene. The mouse line obtained in this way, the "floxed CB1" line, is the starting mouse line for any "conditional" knock-out approach to analyze the behavioural functions of CB1, in the living mouse.

In "conditional" gene inactivation using the Cre/loxP system, the expression of the Cre recombinase is of fundamental importance. The regulatory sequences driving the expression of Cre recombinase in the transgenic mice are currently the main limiting points of this technique. Expression of the recombinase in specific areas or cell types and, possibly, at specific time points is often required. Such characteristics are present in some genes expressed in the brain. As an example, 8.5 kb from the promoter of the CaMK II α gene, encoding the calcium/calmodulin-dependent kinase, can direct Cre expression and, thus, Cre-mediated recombination into specific forebrain areas at postnatal stages in transgenic mice (Tsien et al., 1996; Minichiello et al., 1999). CB1 is widely expressed in the mouse brain (Matsuda et al., 1993; Tsou et al., 1998a; see Chapter 2 and 3), and, in single brain areas, specific cell types appear to coexpress CB1 with various neuronal cell markers. In the mouse forebrain, CB1 is expressed partly in GABAergic and non-GABAergic (glutamatergic) cells (Marsicano & Lutz, 1999; Katona et al., 1999; Chapter 2). Glutamic acid decarboxylase 65K (GAD 65), a specific marker for GABAergic cells, is coexpressed with CB1 in a subset of

cells of hippocampus, neocortex, entorhinal cortex, amygdala and striatum, while the neuropeptide cholecystokinin (CCK), a marker for a subset of both GABAergic and glutamatergic cells, is highly coexpressed with CB1 in hippocampus, neocortex, entorhinal cortex, amygdala, but not in striatum. Thus, transgenic mice carrying Cre recombinase under the control of GAD 65 and CCK, respectively, after crossing with the "floxed CB1" line, could lead to the excision of the gene in specific neuronal subpopulations. "GAD 65-Cre" should lead to the disruption of CB1 in all GABAergic cells, while "CCK-Cre" should lead to the lack of CB1 in both GABAergic and glutamatergic cells, but leave CB1 expression still intact in basal ganglia, thus possibly avoiding locomotor defects that were observed in the CB1 knock out mice of Zimmer et al. (1999).

The major limitation of the Cre/loxP system is the temporal control. Cases like the CaMK II α gene with a perinatal start of expression, are very rare. GAD 65 and CCK are expressed at very early embryonic stages (Pinal & Tobin, 1998; Lay et al., 1999). As a consequence, in GAD 65-Cre or CCK-Cre mice with "floxed CB1", recombination would occur already during development. Therefore, there is the risk of inactivating the gene in neuronal precursors, with the final result of a mouse lacking CB1 in the great majority of neurons, including many cells not expressing GAD 65 or CCK in the adult. Therefore, inducibility of Cre activity is needed to use such such promoters for Cre expression to best effect. Recent reports (Indra et al., 1999; Li et al., 2000) have suggested that a fusion protein of a mutated estrogen receptor ligand binding domain (ET2) and Cre recombinase is an useful tool to regulate the activity of Cre recombinase. This fusion protein is normally inactive, but can be activated by administration of the synthetic steroid hormone tamoxifen. Using this method, a broad range of regulatory sequences is now suitable to drive the expression of Cre recombinase, regardless of its "intrinsic" start of expression. Such "inducible" Cre-expressing transgenic mouse lines under the control of GAD 65 and CCK are in progress to be generated in the laboratory of Dr. Lutz.

As mentioned above in this paragraph, the generation of a "conventional" CB1-deficient mouse line, named CBN, can constitute an useful source of primary tissues where CB1 expression is totally abolished. These tissues can be used in experiments where no spatio-temporal specific disruption of the gene is necessary. One example is the use of primary neuronal cultures, in which the functions of CB1 can be studied by comparing the characteristics of wild type cells and of the CB1-deficient cells in different experimental conditions. Such an approach will be adopted in the next Chapter, where the role of CB1 in neuroprotective properties of cannabinoids will be analysed in oxidative stress experiments.

Chapter 5

Neuroprotective properties of cannabinoids *in vitro*: role of CB1

The work described in this Chapter is in preparation as Marsicano, Moosmann, Behl & Lutz. My contributions were the generation of CB1-expressing cell lines and their characterization, the preparation of primary cerebellar granule cells and the *in vitro* neurotoxicity assays. Bernd Moosmann contributed the biochemical antioxidant assay. Results are given without discriminating the various contributions in order not to disrupt the completeness of the experimental strategy.

5.1 Introduction

Natural and synthetic cannabinoids, as well as endocannabinoids, appear to exert most of their actions in the central nervous system through the "brain type" cannabinoid receptor 1, CB1 (Matsuda et al., 1990; Zimmer et al., 1999; Ledent et al., 1999). However, non CB1-mediated actions of many cannabinoids have been proposed. In particular, the highly lipophilic character of many cannabinoids (see Chapter 1.3.3 "CB1 ligands") could be the basis for a direct interference with cell membranes (Hillard et al., 1985).

Neuroprotective effects have been proposed for natural, synthetic and endogenous cannabinoids in several *in vitro* and *in vivo* neurotoxicity models (Hampson et al., 1998a; Nagayama et al., 1999; Sinor et al., 2000). However, the exact mechanism(s) mediating neuroprotection have not been clearly understood yet. "Classical" and "non classical" cannabinoids, such as Δ^9 -THC and CP 55,940 contain a phenolic ring (Fig 5.1). This chemical structure could confer the antioxidant activity to the lipophilic cannabinoids able to enter the cell membrane (Moosmann & Behl, 1999). Indeed,

"classical" cannabinoids, such as the CB1 agonist Δ^9 -THC and the non CB1-binding component of *Cannabis sativa* cannabidiol, were shown to protect cells from oxidative stress (Hampson et al., 1998a). These observations would indicate a completely receptor-independent action of cannabinoids in this regard. Indeed, for many phenolic natural and synthetic compounds, a protective activity against oxidative stress, independent of any specific receptor-mediated action, has been recently proposed (Moosmann & Behl, 1999). However, CB1 has been implicated in some neuroprotective mechanisms. WIN 55,212-2, a potent CB1 agonist belonging to the family of aminoalkylindoles (see 1.3.3 "CB1 ligands"), exerted a potent neuroprotecting effects in a rat model of global and focal ischaemia (Nagayama et al., 1999). Such an effect appeared to be blocked by the previous administration to the rats of the specific CB1 antagonist SR141716A, pointing to the involvement of CB1 in this effect. Interestingly, in the same report, WIN 55,212-2 was also able to protect cultured rat primary cortical neurons from *in vitro* hypoxia and glucose deprivation, but this effect did not appear to be blocked by CB1 or CB2 antagonists, thus, indicating different mechanisms of action in *in vivo* versus *in vitro* neuroprotection of WIN 55, 212-2.

In the present study, some aspects of neuroprotective effects of cannabinoid compounds, and the involvement of CB1 in these effects, were analyzed. Biochemical assays were performed to test the chemical antioxidant potential of several cannabinoids, and their antioxidant neuroprotective effects were tested in cell lines and rat primary cerebellar granule cell models of oxidative stress. Nine compounds were tested in these experiments: four "classical cannabinoids" (Δ^9 -THC, cannabinol and cannabidiol as *Cannabis sativa* derivatives, and the synthetic HU 210); one synthetic "nonclassical cannabinoid" (CP 55,940); one aminoalkylindole (WIN 55,212-2); one metabolically stable synthetic analogue of anandamide (methanandamide); one CB1 antagonist (SR 141716A); one inhibitor of "anandamide transporter" (AM 404, see

Chapter 1, 1.4.2 "Inactivation of endocannabinoids"). Subsequently, in order to analyze the involvement of CB1 in the neuroprotective effects of various cannabinoid compounds, two genetic approaches were adopted. First, cannabinoids belonging to different subgroups (classed on their ability to activate CB1 and their chemical antioxidant properties) were tested in oxidative stress assays, using cell lines stably transfected with CB1 cDNA and expressing the functional receptor in comparison with parental wild type cells. Second, two cannabinoids, CP 55,940 (antioxidant and CB1 agonist) and WIN 55,212-2 (CB1 agonist, but not antioxidant) were tested as neuroprotective agents in primary granule cell cultures derived either from CB1-knock out mice (CBN mouse line, as described in Chapter 4) or from wild type controls. By these experiments, CB1 was shown not to be directly involved in the mechanism(s) by which antioxidant cannabinoids protect cells from oxidative stress *in vitro*. Moreover, the ability of WIN 55,212-2 to protect primary neurons (but not neuronal cell lines) in *in vitro* oxidative stress assays was also shown not to depend either by intrinsic antioxidant properties of the compound nor by a CB1-mediated mechanism.

5.2 Materials and methods

5.2.1 Chemicals

Cannabinoids were purchased from Sigma, Germany (Δ^9 -THC, cannabinol and cannabidiol), from Tocris, U.S.A. (CP 55,940, WIN 55,212-2, methanandamide, HU 210 and AM 404) and from Sigma/RBI, Germany (SR 141716A). These compounds were prepared as 10 mM stock solutions in 100% ethanol (Δ^9 -THC, cannabinol, cannabidiol and methanandamide) or in 100% DMSO (CP 55,940, WIN 55,212-2, HU 210 and AM 404). Forskolin was purchased from Sigma and was prepared as 10 mM stock solution in DMSO.

5.2.2 Brain lipid oxidation assays

Dissected cerebral cortex of adult Sprague-Dawley rats was homogenized in 3 volumes of degassed lipid buffer (20 mM Tris/HCl, 1 mM MgCl₂, 5 mM KCl, pH 7.4) with a Kontes glass homogenizer (all steps were performed at 4°C). After centrifugation (3000x g, 5 minutes), the pellet was solubilized by sonication in 3 volumes of lipid buffer supplemented with 0.5 M NaCl, incubated for 10 minutes, and centrifuged (100,000x g, 20 minutes). This step was repeated and followed by three washings using 3 volumes of degassed water instead of lipid buffer. Finally, the pellet was resuspended in water at a concentration of 5 mg/ml protein, snap-frozen on dry ice and stored at -80°C.

For the oxidation assay, the rat brain membrane preparation was diluted with PBS to a concentration of 0.6 mg/ml protein and sonicated. Cannabinoids to be tested were added to the 1-ml aliquots at various concentrations (final concentration of ethanol or DMSO was 0.4%) and the oxidative chain reaction was started by adding 50 µM ascorbate and incubated at 37°C. Six hours later, single photon counting was done for 1 minute in a Beckman scintillation counter set in the visible light range. Data were corrected for the baseline photocurrent and normalized to control values.

5.2.3 Cell cultures

Tissue culture plates were purchased from NUNC (Germany). Sterile plastic tubes (50 ml and 15 ml) were purchased from Falcon (Germany).

5.2.3.1 Cell lines

The two neuroblastoma cell lines PC12 and HT22 were used for oxidative stress experiments. Cells were cultured in complete DMEM at 37°C, 5% CO₂ in humidified atmosphere. Complete DMEM consists of Dulbecco's Modified Eagles Medium (DMEM, LIFE Technologies GIBCO, Germany), supplemented with 15% (PC12 cell

line) or 10% (HT22 cell line) foetal calf serum (FCS, LIFE Technologies GIBCO, Germany) and 100 U/ml penicillin and 100 U/ml streptomycin (100x Penicillin/Streptomycin solution, LIFE Technologies GIBCO, Germany). Cells were kept on 10 cm-cell culture dishes and trypsinized prior to experiments. Briefly, after washing twice with sterile phosphate buffer saline (PBS, containing 139.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄-H₂O, 1.8 mM KH₂PO₄, pH 7.4), 3 ml of 0.5% Trypsin/1 mM EDTA (LIFE Technologies GIBCO, Germany) were added to the culture plate, and cells were incubated at 37°C for 10-15 minutes. Cells were pipetted to obtain a single cell suspension, reaction was stopped with complete DMEM, and cells were counted in a haemocytometer. 10⁴ cells/well (PC12 cell line) and 10³ cells/well (HT22 cell line) were plated onto 96-well plates. Each well contained 100 µl of complete DMEM. Cells were incubated overnight and then used for the oxidative stress experiments (see below, 5.2.5 "Oxidative stress assays"). In some experiments, differentiation of PC12 cells was induced by treatment with 1 µM forskolin overnight.

5.2.3.2 *Primary cerebellar granule cells*

Primary cerebellar granule cells were obtained from Sprague-Dawley newborn rats (3 days old) and from newborn mice (3 to 6 days old). For genotyping of the CBN allele (Chapter 4), PCR was performed on DNA derived from tails of 1-2 day old pups. Homozygous wild type (WT) and homozygous CBN/CBN mice (CB1 KO) were used for the experiments. The methods for isolation and culture of primary cerebellar granule cells were identical between rats and mice. Newborn animals were sacrificed by decapitation and cerebella were isolated and put in ice-cold, sterile DMEM containing 100 U/ml penicillin/streptomycin. Using a stereomicroscope which was placed into a cell culture hood, cerebella were dissected free of meninges and put into a 50-ml Falcon tube containing 10 ml of cold DMEM. 10 ml of 0.1% Trypsin/1 mM EDTA were then added to the tube and cerebella were incubated with gentle shaking for 10-20 minutes at

37°C. The tissue was then thoroughly pipetted first with 5-ml plastic pipettes and then with Pasteur glass pipettes. Tissues were centrifuged at 500x g for 4 minutes and then resuspended in 10 ml complete DMEM medium (see previous paragraph). After a further step of disruption by thoroughly pipetting in Pasteur glass pipettes, the tubes were let stand for 5 minutes at room temperature, in order to allow the heaviest debris to sediment. Supernatant, containing cells and small debris, was transferred to a fresh tube and centrifuged. Cell pellet was resuspended in 1 ml of complete DMEM. A drop of the cell suspension was checked under an inverse microscope for the presence of debris and cell clumps. Centrifugation and resuspension were repeated until the quantity of single cells was higher than tissue debris and cell clumps. At this point, cell suspension was counted with a haemocytometer and cells were plated onto poly-L-lysine-treated 96-wells plates, at a density of about 10^6 cells/well. Poly-L-lysine pre-treatment of plates consisted of an incubation for 1-2 hours at room temperature with 50 µl/well of a 10 µg/ml sterile solution of poly-L-lysine (Sigma, Germany), followed by two washes with sterile PBS. After plating, cells were incubated at 37° C in a cell culture incubator. After two days, the cytostatic drug cytosin arabinofuranoside (Sigma, Germany) was added to each well at 10 µM (final concentration), in order to block the overgrowth of fibroblasts and glial cells. After 10-15 days of incubation, the cultures appeared to contain >90% neurons and were ready to be used in oxidative stress experiments (Fig. 5.3C).

5.2.4 CB1 expressing cell lines

5.2.4.1 Cloning of CB1 into an eukaryotic expression vector

Molecular biology methods were as described in Chapter 4. Mouse CB1 cDNA was obtained by PCR using as template clone G23.10 (Chapter 4, 4.3.1.1 "Phage insert cloning"), and the high fidelity *Pfu* polymerase (Stratagene, Germany) was used to minimize the errors caused by PCR. The primer pair was G1-G2 (see Chapter 4, Table

4.1). PCR product was eluted from a gel and subcloned into the *Sma*I site of pBlueScript KS (-) (pKS, Stratagene, U.S.A.), to produce the clone G73.3. The insert was entirely sequenced to check for PCR-induced mistakes. A 1.6 kb *Bam*HI-*Xho*I fragment, containing the whole open reading frame of CB1, was then subcloned into the corresponding sites of the eukaryotic expression vector pcDNA3 (Invitrogen, Germany) to generate clone G160.1, which was then used for the stable transfection of CB1 in PC12 and HT22 cells.

5.2.4.2 *Electroporation of cells and selection of resistant clones*

Electroporation protocols were identical for PC12 or HT22 cell lines. A confluent 10-cm cell culture plate was trypsinized as described above (5.2.3 "Cell cultures"). Single cells were transferred into a 50-ml Falcon tube and centrifuged for 4 minutes at 200x g. Cell pellet was resuspended in 390 μ l of electroporation buffer (50 mM K_2HPO_4 , 63.9 mM K_2HPO_4 , 16.17 mM potassium acetate, pH 7.35). 10 μ l of 1 M $MgSO_4$ were freshly added to the cell suspension. Cell suspension was carefully mixed with 10 μ l of a solution containing 1 μ g/ μ l of *Not*I-linearized plasmid G160.1. The mixture was then transferred to an electroporation cuvette (BioRad, Germany). Electroporation was performed in a Gene Pulser electroporation apparatus (BioRad, Germany) at the following settings: 0.3 kV, 500 μ F. After electroporation, cells were kept on ice for 5 minutes and then plated onto a 10-cm cell culture plate with complete DMEM. Cells were incubated for two days and medium was changed every 24 hours to remove dead cells. Cells were then split at dilutions 1:3 (PC12) and 1:4 (HT22), and selection with geneticin (G418, LIFE Technologies GIBCO, Germany) was started. Preliminary dose-response experiments on parental cells showed that 3.5 mg/ml and 2.5 mg/ml were the optimal concentration of G418 to induce cell death in HT22 and PC12 cells, respectively (data not shown). After 8-10 days selection, resistant cell clones became readily visible and were picked with the aid of sterile 200 μ l pipette tips. Clones were transferred to 24-

well plates containing 0.5 ml of complete DMEM. Cells were pipetted up and down to disrupt the colony and obtain a single cell suspension. Clones were grown until they formed confluent monolayers (medium was changed every two days). Confluent clones were trypsinized and transferred to 6-cm cell culture plates. After reaching confluence, cells were split to a 10-cm cell culture plate. At confluency, cells were split at dilution 1:3. After 2-3 days, cells from two plates were frozen (see Chapter 4, 4.2.2.3 "Electroporation of ESCs, antibiotic selection, picking and expansion of resistant clones"), whereas one plate was used for RNA extraction. After Northern blot screening, positive cell clones were thawed and expanded to generate several aliquots of frozen cells.

5.2.4.3 Northern blot analysis of resistant clones

RNA extraction from confluent 10-cm cell culture plates was performed using the PeqGold RNAPure kit (PeqLab, Germany), following the manufacturer's instructions. All plasticware used for Northern blot analysis was treated with 1 M NaOH and subsequent rinses with RNase-free water to get rid of RNAase; all solutions were prepared RNase-free by DEPC treatment and autoclaving. RNA extracts were electrophoresed in a gel containing 1x running buffer, 1% agarose and 6% formaldehyde. Running buffer was prepared as a 10x solution containing 200 mM 3-(N-morpholino) propane sulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0. Samples were prepared by mixing on ice 4 µl of RNA (5 µg/µl) and 16 µl denaturing mix. After incubation at 55°C for 15 minutes, samples were chilled on ice and 2 µl of loading buffer were added. Denaturing mix consists of 200 µl formamide, 70 µl of 37% formaldehyde, 40 µl of 10x running buffer and 10 µl H₂O. Loading buffer was prepared as a 10x stock solution containing 50% glycerol, 1mM EDTA, 0.2% bromophenol blue. After electrophoresis, capillary blot was performed in 10x SSC, as described in Sambrook et al. (1989) to transfer RNA onto Hybond NX filters (Amersham, USA). Hybridization was performed using the fragment CB1HIII as probe (see Chapter 4,

Fig. 4.4). Probe labeling was performed using the Random Primers DNA Labeling System kit (LIFE Technologies GIBCO, Germany) following the manufacturer's recommendations. Hybridization was performed as Southern hybridization (see Chapter 4, 4.2.1.5.2 "Hybridization") with the only differences that pre-hybridization, hybridization and washing were at 68°C. Washed membranes were exposed 1-3 days onto autoradiography films (DuPont, U.S.A.).

5.2.5 Oxidative stress assays

The day before the experiment, cell lines were plated onto 96-well plates as described above (5.2.3 "Cell cultures"). Cannabinoids were prepared as pre-dilutions in ethanol or DMSO, and 1 µl of each pre-dilution was added to each well containing the cells and 100 µl of medium. Controls were obtained by adding 1 µl of 100% ethanol or DMSO to each control well. Experiments were performed in triplicate or quadruplicate and were repeated at least twice for each cell clone or primary cell culture. After adding the drug, cells were incubated overnight, and then, different concentrations of H₂O₂ (prepared in sterile water) were added, ranging from 60 µM to 250 µM. The volume of the H₂O₂ pre-dilution added to each well was 1 to 3 µl. After overnight incubation, 10 µl of a 5 mg/ml solution in water of dimethylthiazolyl-diphenyl-tetrazolium bromide (MTT, Sigma, Germany) were added to each well. MTT, which is normally yellow, is transformed in the mitochondria of living cells into the insoluble precipitate formazan, that appears blue. After 4 hours MTT incubation at 37°C, 100 µl of cell lysis solution (45% dimethylformamide, 10% SDS, pH 4.2) were added to each well. Lysis was allowed overnight at room temperature and the day after the plates were read with Dynatec microplate reader (Germany) set at a 570 nm wave length. Cell death was indicated by a decreased MTT reaction and thus by a decreased absorption at 570 nm. Values in the wells where both vehicle (ethanol or DMSO) and H₂O₂ were added (maximal death) were compared to the values of the wells where only vehicle was added

(maximal survival, 100%). The fraction between these values gave the maximal percentage of death in the experiment. Only experiments where a maximal cell death percentage of >70% (i.e. <30% survival) was obtained, were considered in the Results section. In order to compensate any proliferative or antiproliferative effects of the drugs, values derived from wells containing the different concentrations of cannabinoids and H₂O₂ were normalized to the corresponding concentration of drug in the absence of H₂O₂. To better compare the results of different experiments, data are given as percentages of the maximal theoretical protection (100%, corresponding to the value in vehicle-treated wells, without toxin), considering as 0% the maximal cellular death. Therefore, each value is calculated as follows:

$$\frac{DS - TCS}{MP - TCS} \times 100,$$

where DS is the drug-induced percent of survival, TCS is the cell survival in the presence of H₂O₂ alone (with <30% as the limit for the acceptance as a successful experiment), and MP is the theoretical maximal protection (100%, corresponding to vehicle-treated cells).

5.3 Results

5.3.1 Cannabinoids as antioxidative and neuroprotective agents

Cannabinoid compounds are generally classified into four different chemical groups, based on their structural characteristics (Pertwee, 1997; Fig 1.4). Regarding putative neuroprotective properties, cannabinoids can also be classified into three groups, based on whether or not they bind to cannabinoid receptors and whether or not they have a phenolic structure: i) phenolic compounds that do not bind CB1 (Fig. 5.1A); ii) non phenolic compounds that bind CB1 (Fig 5.1B); iii) phenolic compounds that bind CB1 (Fig. 5.1C).

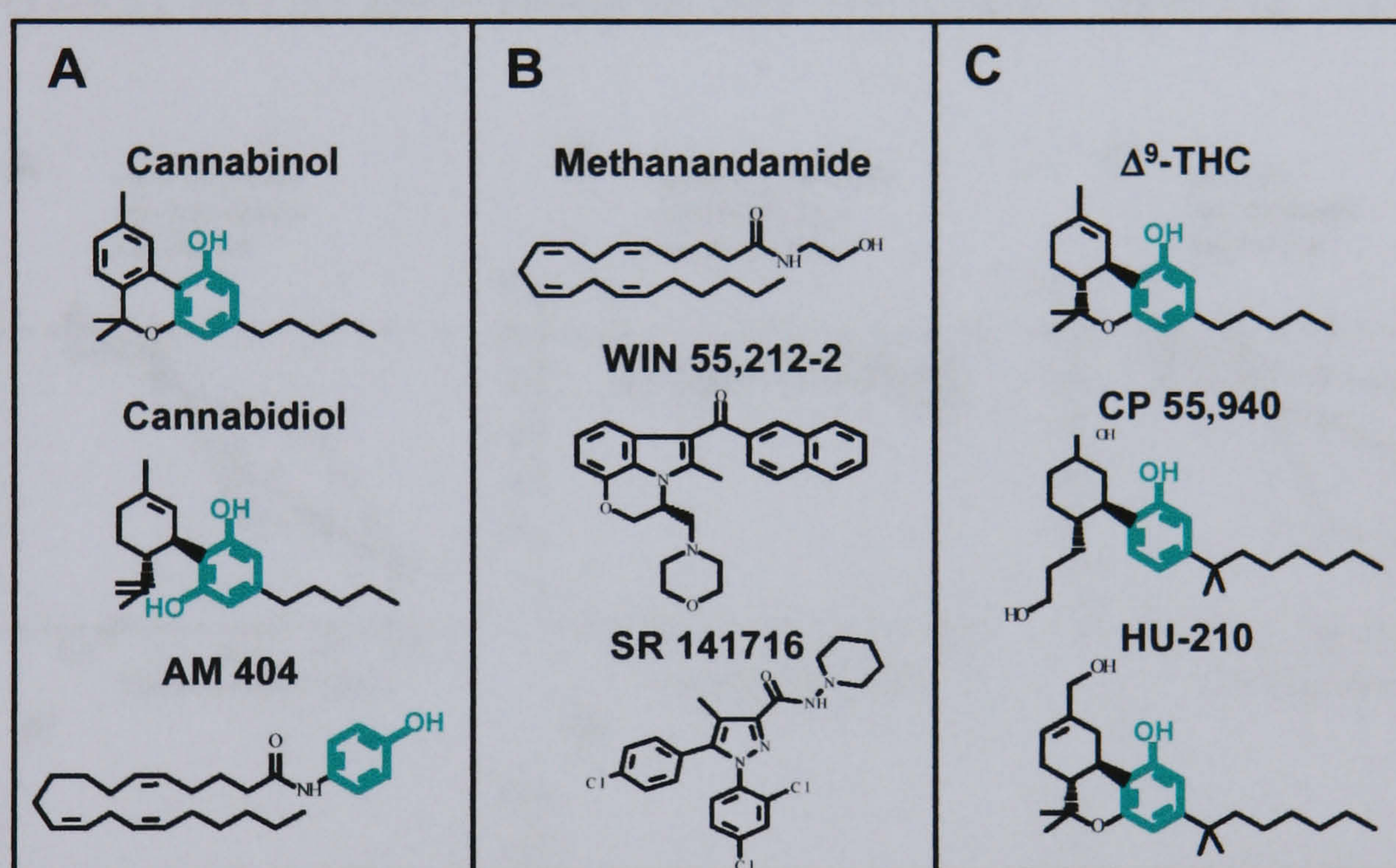


Fig 5.1. Classification of cannabinoids on the basis of CB1 binding and presence of phenolic moieties (in green). A. Phenolic cannabinoids with no or very low affinity for CB1. B. Nonphenolic compounds with high affinity for CB1. C. Phenolic cannabinoids with high affinity for CB1.

Accordingly, Fig 5.2A, B and C show the antioxidant properties of various cannabinoids and cannabimimetics. Compounds containing a phenolic group, such as cannabinol, cannabidiol, AM 404 (not binding CB1, Fig. 5.2A), and Δ^9 -THC, CP 55,940 and HU 210 (binding CB1, Fig. 5.2C) were shown to be potent antioxidants in a brain lipid oxidation assay. On the other hand, cannabinoids not containing a

phenolic ring, such as methanandamide (a stable analogue of anandamide), WIN 55,212-2 and SR 141716A did not show any antioxidant activity in the same assay (Fig 5.2B). Oxidative stress experiments in HT22 cells generally reflect the chemical antioxidant properties of the tested compounds, e.g. oestrogenic drugs (Bernd Moosmann, personal communication). Therefore, this notion was tested here for cannabinoids. Indeed, the same compounds that showed biochemical antioxidant properties resulted also in potent protection for H₂O₂-induced oxidative stress in HT22 cells (Fig. 5.2A', C'), whereas the non phenolic compounds, regardless of whether they were CB1 agonists (methanandamide and WIN 55,212-2) or CB1 antagonists (SR 141716A), were not able to protect the cells from oxidative stress (Fig. 5.2B').

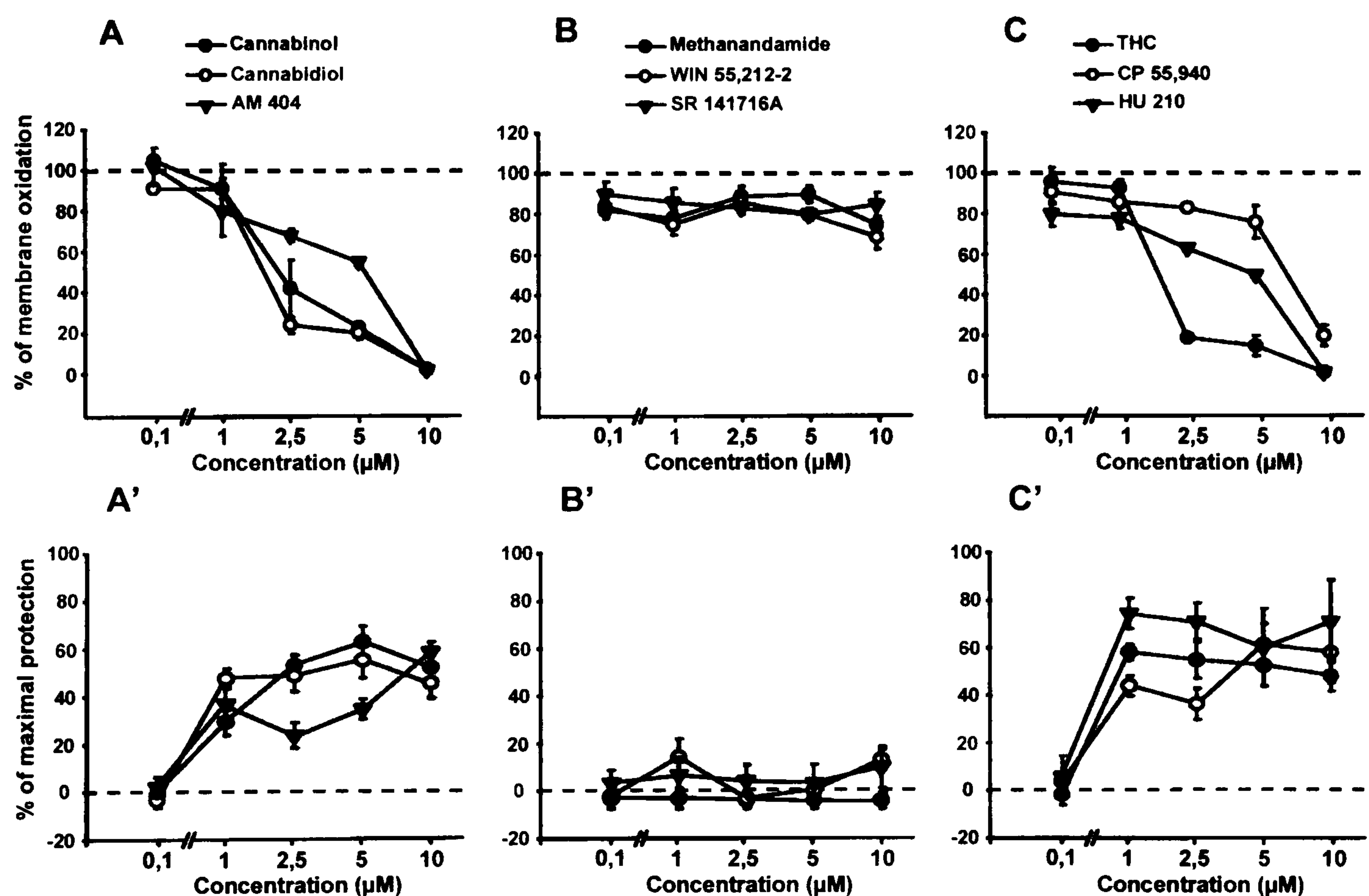


Fig 5.2. Antioxidant properties of cannabinoids and protection against oxidative stress in HT22 cells. A, B, C, percentages of inhibition of oxidation of brain lipids induced by 50 μM ascorbate. 100% indicates maximal oxidation in the absence of any cannabinoid. A', B', C', percentages of protection against oxidative stress in HT22 cells, induced by 120 μM of H₂O₂. 0% indicates the cell death in the absence of cannabinoids. 100% indicates the maximal possible protection against oxidative stress. Data are indicated as ± s.e.m.

These results seem to indicate the antioxidant properties of cannabinoids as the main mean through which they exert neuroprotection in oxidative stress assays. However, neither HT22 nor PC12 cells express CB1 (see below, Fig. 5.4). Therefore, from these results, it cannot be excluded that cannabinoid-mediated neuroprotection is dependent in part on a receptor-dependent mechanism. Thus, as a source of CB1-expressing neurons, rat primary cerebellar granule cells cultures were assessed and tested in similar oxidative stress paradigms.

5.3.2 Cannabinoids are neuroprotective in rat cerebellar granule cells

Neuroprotective properties of cannabinoids were then assessed in oxidative stress assays carried out on cultured primary cerebellar granule cells which are known to contain CB1 protein (e.g. Hillard et al., 1999). Four CB1 agonists were chosen for these tests, namely the two non phenolic compounds methanandamide and WIN 55,212-2 and the two phenolic antioxidant compounds CP 55,940 and HU 210. Fig. 5.3A and C show that CP 55,940 and HU 210 possess similar neuroprotective potentials as in HT22 cells (Fig. 5.2C, C'). These results indicate that phenolic cannabinoids are able to protect primary granule cells in *in vitro* oxidative stress assays. In contrast, the results with the other two compounds, WIN 55,212-2 and methanandamide, seem to indicate different mechanisms of cannabinoid protection in neuronal cell lines and cultured primary neurons. These compounds belong to the group of CB1-binding molecules that are neither phenolic (Fig 5.1B), nor antioxidant or protecting HT22 cells from oxidative stress (Fig 5.2B, B'). Fig. 5.3B shows that methanandamide does not appear to have any neuroprotective effect on granular neurons at any of the concentrations tested, whereas WIN 55,212-2 seems to be able to protect granular neurons from oxidative stress, as protection values of $43 \pm 0.5\%$ and $25 \pm 0.6\%$ were reached at $1 \mu\text{M}$ and $10 \mu\text{M}$, respectively.

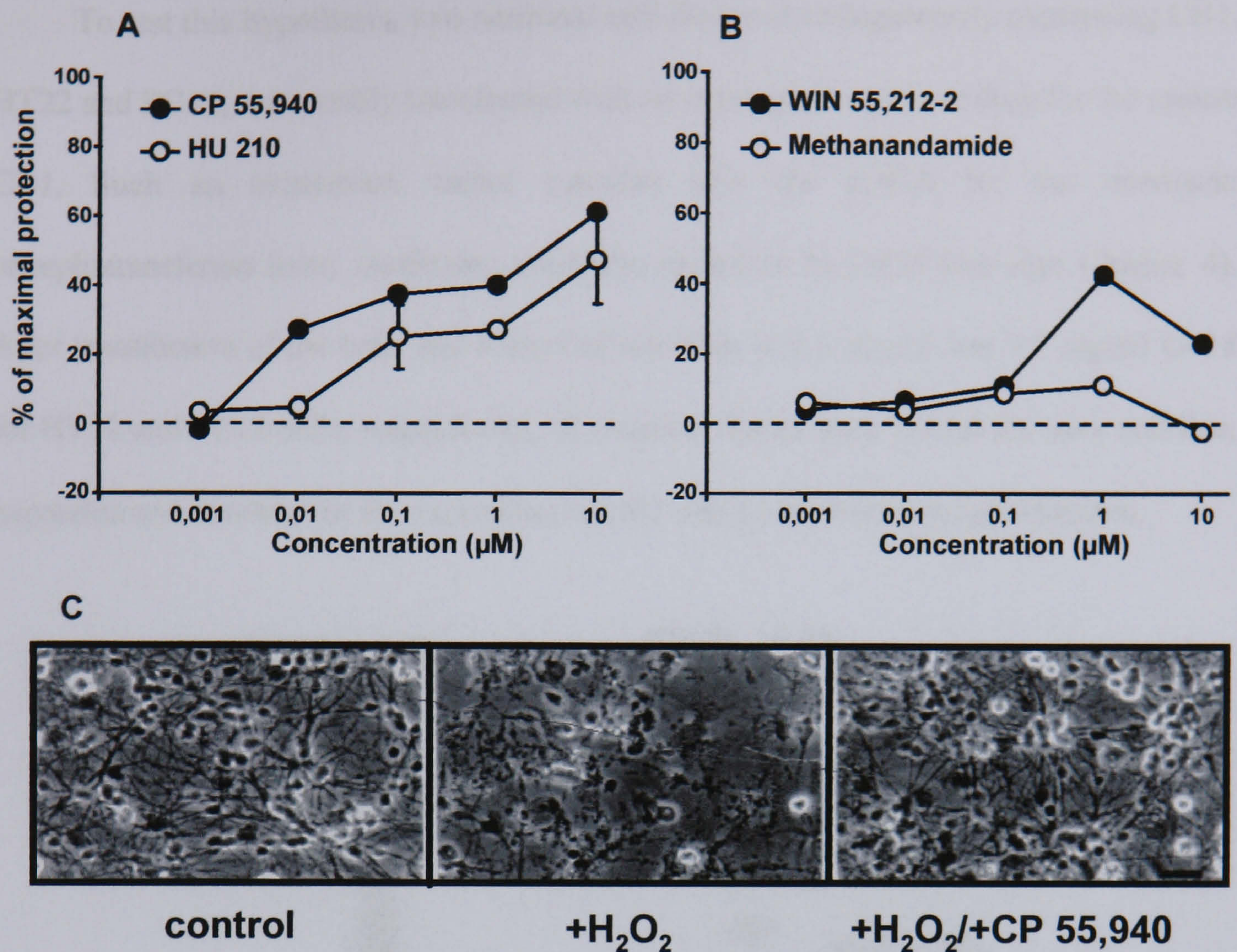


Fig. 5.3. Cannabinoid-mediated neuroprotection in oxidative stress assays on cultured primary rat cerebellar granule cells. A. Neuroprotective effects of CP 55,940 and HU 210 (phenolic antioxidant CB1 agonists). B. Neuroprotective effects of WIN 55,212-2 and methanandamide (non phenolic CB1 agonists) C. Phase contrast micrographs showing examples of CP 55,940-mediated neuroprotection in cultured primary rat cerebellar granule cells. In A and B, results are presented as in Fig. 5.2A', B', C'.

These results indicate that in oxidative stress assays conducted with primary cerebellar granule cells, the protective properties of some cannabinoids cannot be only attributed to their biochemical antioxidant actions, and hence, the question remains of whether a receptor-mediated mechanism exists.

5.3.3 Cannabinoid-mediated neuroprotection in neuronal cell lines expressing CB1

Cerebellar granule cells express CB1 (Matsuda et al, 1993; see also Chapter 1). Therefore, it is possible to hypothesize that the presence of the receptor may account for the differences in *in vitro* neuroprotective properties of different cannabinoids (i.e. WIN 55,212-2) observed in neuronal cell lines as compared to primary neuronal cell cultures.

To test this hypothesis, two neuronal cell lines not endogenously expressing CB1, HT22 and PC12, were stably transfected with an expression vector coding for the mouse CB1. Such an expression vector contains also the cDNA for the neomycin phosphotransferase gene, conferring antibiotic resistance to G418 (see also Chapter 4). After transfection of the cells and 8 days of selection in 2.5 mg/ml and 3.5 mg/ml G418 for HT22 and PC12 cells, respectively, 18 resistant clones were picked for each cell line, expanded and checked for the expression of CB1 mRNA by Northern hybridization.

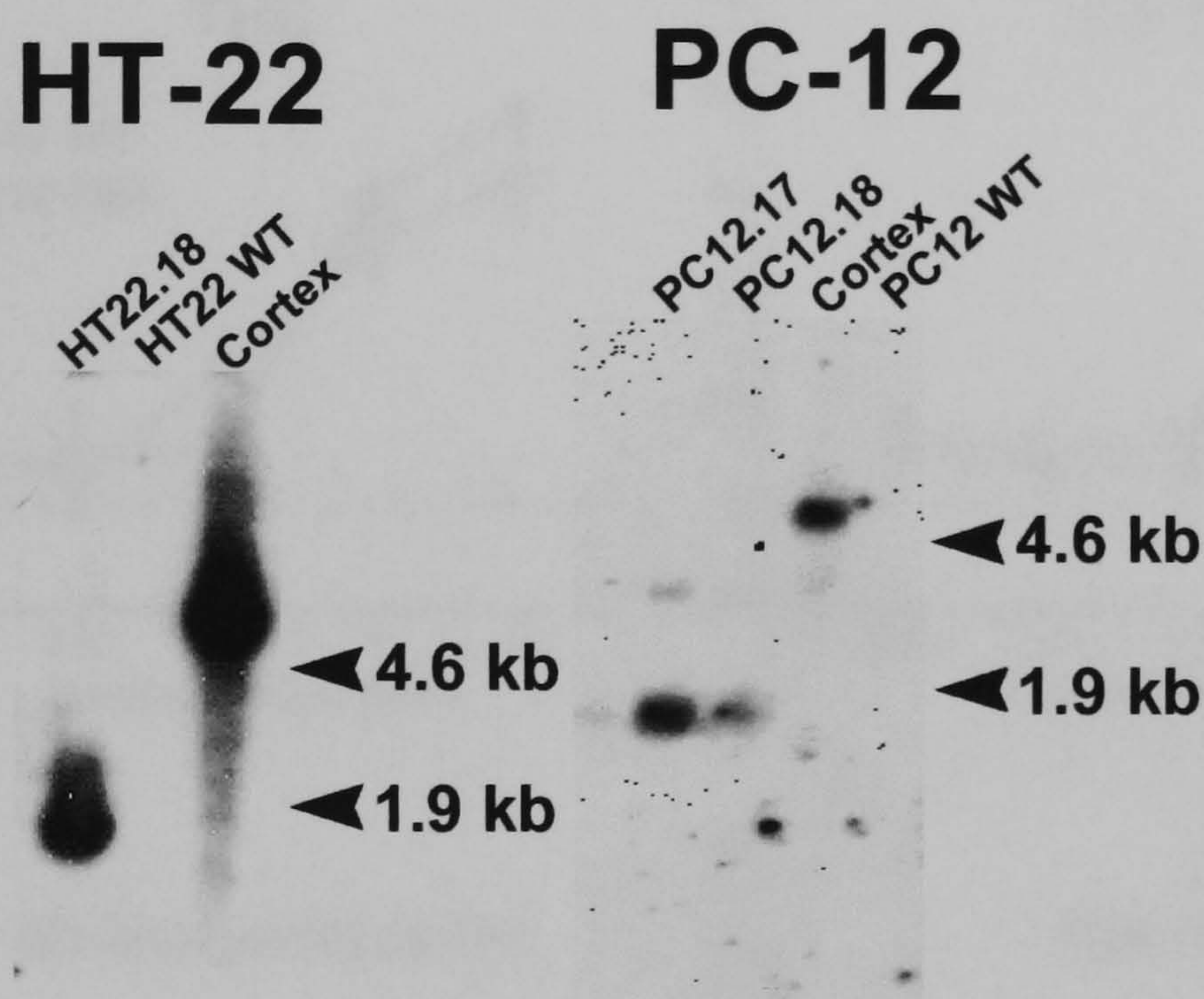


Fig 5.4. Northern blot analysis of HT22 and PC12 cell clones, after stable transfection with the expression vector G160.1, containing the mouse CB1 cDNA. Ribosomal RNA was used as molecular weight marker (28S, approximately 4.6 kb; 18 S, approximately 1.9 kb). The fragment CB1HIII (see Chapter 4, Fig. 4.4) was used as probe. Mouse cortex RNA, containing high levels of CB1 RNA (about 6.0 kb), was used as positive control. Negative controls were cell clones transfected with the empty expression vector (WT). Positive signals in clones HT22.18, PC12.17 and PC12.18 correspond to a band of about 1.6-1.7 kb, consistent with the expected size.

Cells transfected with an empty vector were used as negative controls (termed as PC12 WT and HT22 WT). Fig 5.4 shows the results of Northern blots of HT22 and PC12 RNA extracts, probed with the fragment CB1HIII (see Chapter 4, Fig. 4.4). The positive control was RNA from mouse cortex. Hybridization signals were detected at approximately 6.0 kb for cortex RNA, as previously described (Matsuda, 1997) and at

approximately 1.6 kb for many G418-resistant CB1-transfected clones. As expected, no band was present in G418-resistant clones transfected with the empty vector (PC12 WT and HT22 WT). Clones HT22.18 and PC12.17 (PC12 CB1 in Fig. 5.5) with their respective parental negative controls (WT) were used for further experiments (Fig. 5.4). Fig. 5.5 shows the neuroprotective effects of four CB1 agonists in PC12 cells either expressing (PC12 CB1) or not expressing (PC12 WT) the receptor.

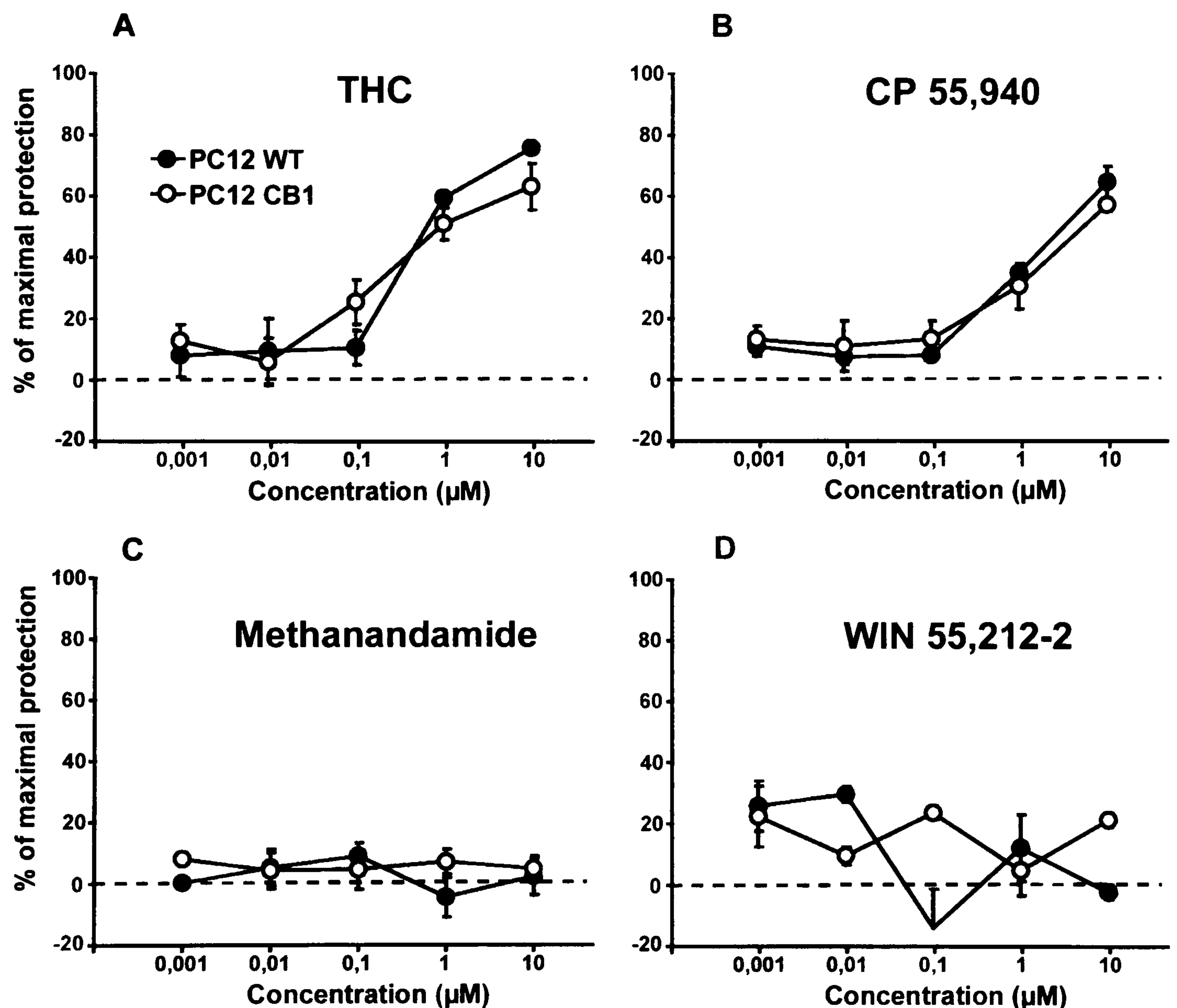


Fig. 5.5. Cannabinoid-mediated neuroprotection in *in vitro* oxidative stress assays on PC12 cells expressing CB1 (PC12 CB1) and not expressing CB1 (PC12 WT). Results are presented as in Fig. 5.2A', B', C'. Note the lack of difference between the two cellular genotypes.

The four CB1 agonists to be tested were chosen in light of their previously determined effects in the antioxidant assay and in the oxidative stress assays in HT22 cells and primary cerebellar granule cells cultures (Fig. 5.2 and 5.3). Δ^9 -THC and CP 55,940

were used as prototypes of the phenolic antioxidant group, while methanandamide and WIN 55,212-2 represented non phenolic-non antioxidant compounds (Fig. 5.2). As shown in Fig 5.5A and B, the two phenolic compounds Δ^9 -THC and CP 55,940 were able to protect PC12 cells up to values of 60-70% and 55-60%, respectively. However, no difference was observed between the wild type (PC12 WT) and the CB1-expressing (PC12 CB1) clones. The dose-response curves were almost identical, thus indicating that the presence of CB1 was altering neither the efficacy nor the potency of the drugs. Once again, methanandamide (Fig. 5.5C) showed no ability to protect cells from oxidative stress, neither in the absence nor in the presence of CB1. Also WIN 55,212-2 (Fig. 5.5D), despite some apparent effect at low concentrations, could not show any reliable and significant protective effect even at concentrations as high as 10 μ M. Similar experiments using HT22 WT and HT22.18 gave very similar values as the ones depicted in Fig. 5.2B' and C', and no difference was observed between the two different genotypes (data not shown). These observations indicate that CB1 is not required for the protective effects of cannabinoids in *in vitro* oxidative stress assays in neuronal cell lines. Neither WIN 55,212-2, which is able to protect primary cerebellar granule cells, did show any clear effect in CB1-expressing cell lines.

5.3.4 Cannabinoid-mediated neuroprotection in primary cerebellar granule cells from CB1 knock out mice

WIN 55,212-2 was shown to protect rat primary granule cells from oxidative stress (Fig. 5.3B), but not neuronal cell lines regardless the presence of CB1 (Fig. 5.2B' and Fig. 5.5D). It is possible that in primary cultures, the presence of CB1 is associated with other factors that are absent in neuronal cell lines. If this is the case, WIN 55,940 should not be able to protect cultured primary granule cells that do not contain CB1. In order to test this hypothesis, primary cerebellar granule cell cultures were assessed from WT and CB1 knock-out mice (CBN/CBN, see Chapter 4).

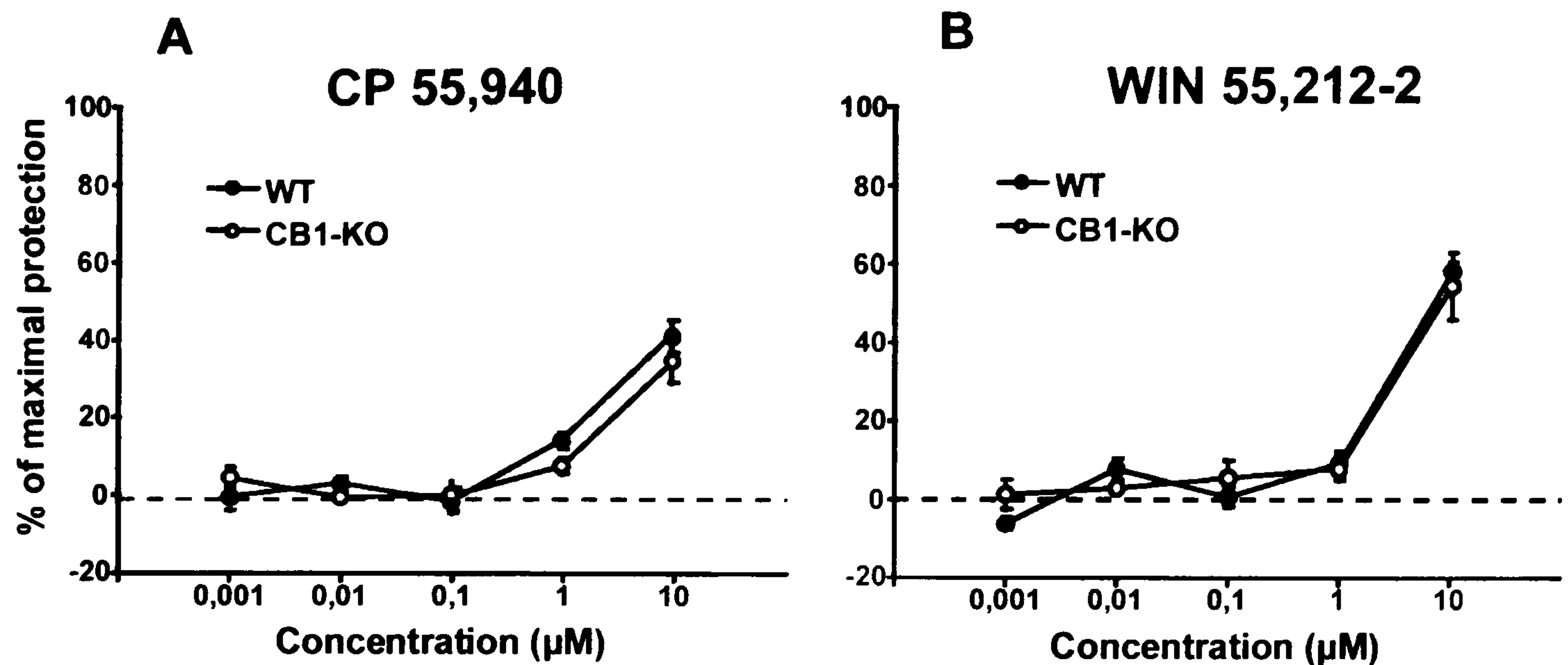


Fig. 5.6. Cannabinoid-mediated neuroprotection in *in vitro* oxidative stress assays on primary cerebellar granule cell cultures derived from wild type mice (WT) and homozygous CBN/CBN littermates (CB1 KO, see Chapter 4). Results are presented as in Fig. 5.2A', B', C'. Note the lack of difference between the two genotypes and the protection caused by WIN 55,212-2.

CP 55,940 was used as phenolic antioxidant cannabinoid, and its effects were compared to WIN 55,212-2. Fig. 5.6A shows that CP 55,940 was able to protect WT and CB1-KO cells as much as 40%, whereas WIN 55,212-2 (Fig. 5.6B) showed a protection to almost 60%. However, no difference was observed between WT and CB1-KO cells. These results indicate that CB1 is not necessary for the neuroprotective activity of CP 55,940 and WIN 55,212-2 in the *in vitro* oxidative stress assays using H₂O₂.

5.3.5 Cannabinoid-mediated neuroprotection in differentiated PC12 cells

Although at high concentrations, WIN 55,212-2 is able to protect primary granule cells in a CB1-independent manner, but not neuronal cell lines. Neuronal cell lines share many characteristics with neurons, but have also some important differences. One of the most important is that neuroblastoma cell lines consists of proliferating and relatively undifferentiated cells, whereas mature neurons do not proliferate. It is, therefore, possible that the differentiation state of the cells play a role in the differential protective effects of WIN 55,212-2. In order to test this hypothesis, neuronal differentiation was induced in PC12 cells by treatment with forskolin (Caillaud et al., 1995).

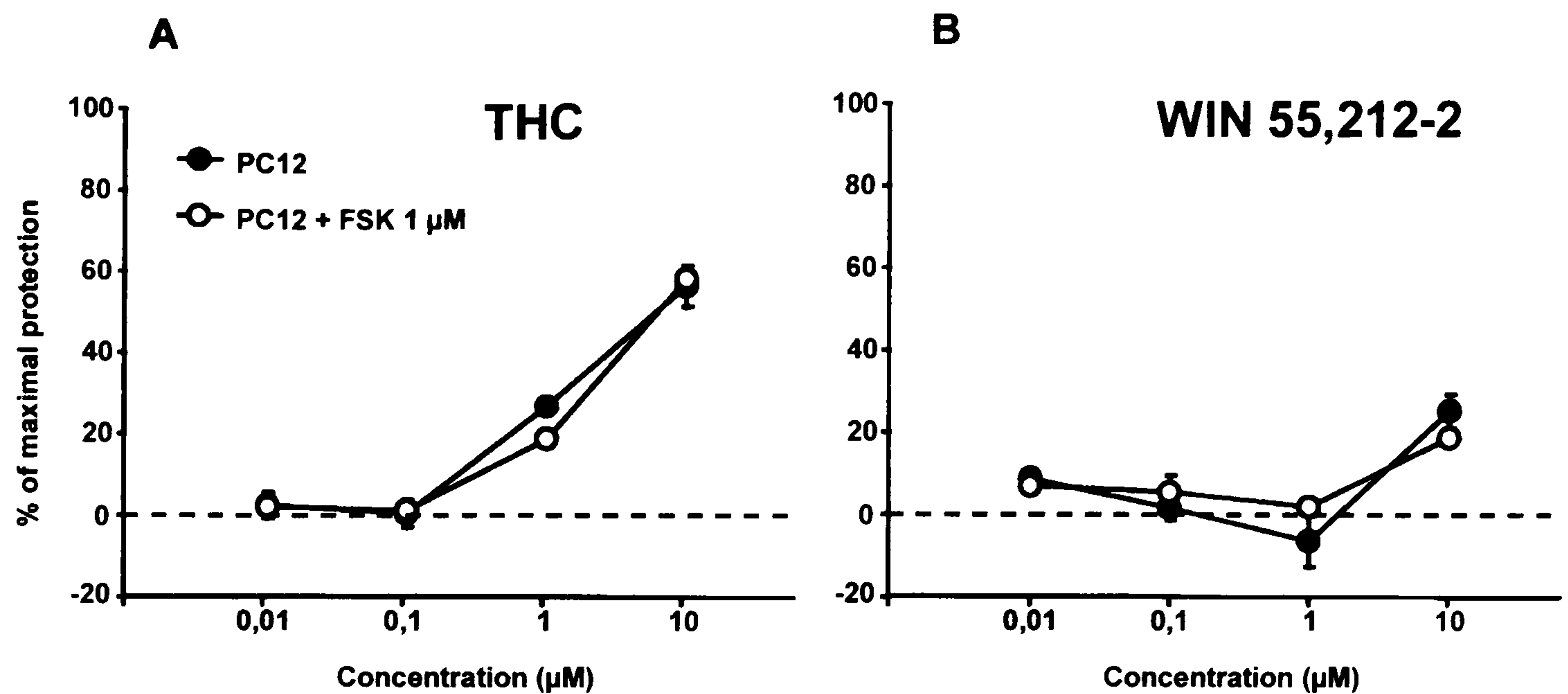


Fig. 5.7. Protection against oxidative stress in undifferentiated (PC12) and differentiated (PC12 + 1 μM forskolin) PC12 cells. Results are given as in Fig. 5.2 A', B', C'.

After overnight treatment with 1 μM forskolin, PC12 cells showed a neuron-like morphology (not shown). Fig. 5.7A shows that Δ^9 -THC was equally able to protect undifferentiated and differentiated PC12 cells. However, WIN 55,212-2 was not able to protect either of the two cell types against oxidative stress (Fig. 5.7B). These results indicate that the differentiation state is unlikely to account for the differential neuroprotective effects of WIN 55,212-2 on PC12 cell line and on primary neurons.

5.4 Discussion

In this Chapter, the neuroprotective properties of cannabinoids were analysed in *in vitro* oxidative stress assays. Oxidative stress represents one of the major events that occurs during neurodegeneration in many neurological diseases (Behl, 1999a; Browne et al., 1999; Fahn & Cohen, 1992). Therefore, drugs which are able to inhibit these processes are ideal candidate for the treatment of such diseases. Chemical antioxidants, e.g. vitamin E (Behl, 1999b), are examples of these neuroprotective drugs. However, oxidative stress is accompanied by a series of complex intracellular events that contribute to the cell death and that can be modulated also by means other than a simple chemical antioxidant activity. As an example, oxidative stress and excessive activation of glutamate receptors, with the consequent excessive intracellular concentration of Ca^{2+} represent sequential and

interacting processes that provide a final common pathway for cell vulnerability in the brain (Coyle & Puttfarcken, 1993). Many cannabinoids have typical structural features of antioxidants, and on the other hand, through CB1 activation, they are able to inhibit excitability of the cells, by increasing K^+ and decreasing Ca^{2+} permeability (see Chapter 1, 1.3.1 "Mechanisms of action" and references therein). Therefore, they could exert neuroprotective activities through different mechanisms and thus, possess very interesting therapeutic potentials for several neurodegenerative diseases.

Several cannabinoids were tested in a biochemical antioxidant assay, and, as expected, phenolic compounds (Δ^9 -THC, cannabitol, cannabidiol, CP 55,940, HU 210 and AM 404), were found to be potent antioxidant. These observations were confirmed by *in vitro* oxidative stress experiments, by using two neuronal cell lines, PC12 and HT22, and primary cerebellar granule cells: antioxidant phenolic cannabinoids were also protective against oxidative stress on the cells. However, one compound, WIN 55,212-2, a potent CB1 agonist belonging to the family of aminoalkylindoles, was able to protect primary neurons but not cell lines. This observation is interesting, also in view of the fact that this compound is not a phenolic antioxidant. Other mechanism(s) should, therefore, be triggered by the drug in primary neurons that are not present in cell lines. The most obvious candidate for this differential protective mechanism appeared to be the presence of CB1 in cerebellar primary neurons, but not in neuronal cell lines. One way to test this hypothesis would be to use the specific CB1 antagonist SR141716A in the same neurotoxicity assays, in combination with the CB1 agonists (Nagayama et al., 1999). However, SR141716A was shown to exert, both *in vivo* and *in vitro*, actions different from the simple antagonistic effect at CB1 receptors. In CB1-transfected cells and in endogenously CB1-expressing neuronal cell lines, SR141716A was shown to act as an inverse agonist (Shire et al., 1999; Meschler et al., 2000) and, furthermore, in primary cerebellar granule cells, SR141716A was recently shown to exert different

effects on cannabinoid-mediated inhibition of Ca^{2+} mobility in different subcellular portions of the neurons. A mere antagonistic effect was observed at CB1 receptors located in the neurites, whereas a paradoxical "agonist-like" inhibition of Ca^{2+} influx was observed in the soma of the neurons (Hillard et al., 2000). A direct consequence of these observations is that, at least in *in vitro* experiments, the use of SR141716A cannot be considered always a reliable mean to detect receptor-mediated actions of CB1 agonists. Therefore, the involvement of CB1 in WIN 55,212-2-mediated neuroprotection was checked by two genetic approaches, i.e. in heterologously CB1-expressing cell lines as compared to parental lines and in primary cerebellar neurons derived from wild type and CB1-deficient mice (CBN/CBN, see Chapter 4). However, unexpectedly, no differences were observed in the neuroprotective activity of the drug in presence or in absence of CB1. Another difference between primary neurons and neuronal cell lines is the differentiation state. It is possible that WIN 55,212-2 exert its neuroprotective actions only in differentiated postmitotic neurons and not in proliferating cell lines. However, even in forskolin-induced differentiated PC12 cells, WIN 55,212-2 was not able to exert the same neuroprotective actions as observed in primary neurons.

An intriguing hypothesis that could explain the effects of WIN 55,212-2 in neuronal protection of primary neurons is that these actions are mediated by another receptor, distinct from CB1. WIN 55,212-2 is a potent agonist of CB1, but is also able to bind and activate the "peripheral" cannabinoid receptor, CB2 (Pertwee, 1997). However, despite discordant reports (Skaper et al., 1996), CB2 does not appear to be functionally present in cerebellum (Griffin et al., 1999). Interestingly, recent results showed that WIN 55,212-2 is able to stimulate G proteins in cerebral membranes derived from CB1-deficient mice, thus strongly suggesting the existence of a novel "cannabinoid receptor" (Breivogel et al., 2000). Therefore, it could be argued that this "third" receptor might be

the responsible for neuroprotective effects of WIN 55,212-2. However, preliminary experiments conducted in our laboratory to test the ability of the drug to inhibit forskolin-induced increases in cAMP production in primary cerebellar neurons from wild type and CBN/CBN mice, revealed that WIN 55,212-2 is able to inhibit cAMP accumulation in presence of CB1 but has no effect in mutant neurons. It appears, therefore, unlikely that the "third" receptor uses a similar signal transduction pathway to CB1. However, G proteins can trigger also many other intracellular pathways. Experiments in this direction, in relation to the mechanisms underlying the neuroprotective properties of WIN 55,212-2 and the existence of a novel signal transduction pathway stimulated by the drug, are planned in the laboratories of Dr Lutz and Dr Behl.

H₂O₂-induced oxidative stress *in vitro* is a relatively simple paradigm of neurodegeneration that can provide useful information about the neuroprotective aspects of several drugs. However, the *in vivo* situation, during neurodegenerative conditions induced by brain damage or diseases, is more complex than any *in vitro* models. As an example, cannabinoids, and WIN 55,212-2 in particular, were shown to act differentially in similar neurodegenerative models *in vivo* or *in vitro* (Nagayama et al., 1999). Therefore, the apparent CB1-independent mechanism of protection of cannabinoids as observed in this *in vitro* study needs to be confirmed *in vivo*. In other words, an involvement of CB1 in neuroprotection mechanisms during neurodegeneration, as shown, for example, by the increased CB1 expression in particular brain areas during induced ischaemia in rats (Jin et al., 2000), cannot be excluded. Moreover, it could be proposed that the increased mortality observed by Zimmer et al. (1999) in CB1-deficient mice might be due to a general decrease of neuroprotective mechanisms in which CB1 might play an important role *in vivo*. Therefore, experiments of *in vivo* neurodegeneration induced in different ways are planned, using CBN/CBN and/or

"conditional" CB1 mutants (see Chapter 4) mice compared with wild type controls.

In conclusion, in this Chapter, some aspects of neuroprotective properties of cannabinoids were analysed. The most important results are the purely antioxidant and CB1-independent mechanism in neuroprotection in these particular *in vitro* paradigms using neuroblastoma cell lines, and the non antioxidant and CB1-independent neuroprotection induced by WIN 55,212-2 in cerebellar granule cells. These results would indicate the existence of a novel signal transduction pathway triggered by WIN 55,212-2 in cerebellar neurons.

Conclusions and future perspectives

The work described in this thesis was designed to better understand some of the physiological functions of the "brain type" cannabinoid receptor CB1 and of the cannabinoid system in the mouse central nervous system. Neurochemical and neuroanatomical aspects were analysed in Chapter 2 and 3, with some interesting findings regarding the distribution and the expression of CB1 at cellular level. These findings further underline the potential central role of the cannabinoid system as a modulator of many of the brain functions in physiological and pathological conditions. Neurochemical and neuroanatomical results would indicate that the cannabinoid system might potentially influence both excitatory and inhibitory pathways in the brain and might establish many functional interactions with other signalling systems.

A functional analysis of one of these putative interactions is presented in Chapter 3, where the effects of Δ^9 -THC were analysed in mice with a disrupted neuronal nitric oxide synthase (nNOS) gene. Results demonstrated that locomotor and hypothermic effects of the cannabinoid drug are absent in the mutant animals as compared to wild type controls, thus indicating a central key function of the nitric oxide (NO) pathway in these cannabinoid effects. Moreover, mutant animals have a selective reduction of CB1 mRNA expression in particular brain areas, thus revealing a putative cross-talk between NO and cannabinoid system also at the level of expression control. These studies are still ongoing in Dr. Lutz's laboratory, with the discovery of decreased levels of CB1 also in hippocampi of nNOS mutant animals. This will open new perspectives in the analysis of behavioural effects of cannabinoids in this mutant strain, regarding hippocampal-dependent learning and memory paradigms. Moreover, in collaboration with other groups in the Institute and outside, experiments are planned to analyse hippocampal electrophysiological responses to cannabinoids in nNOS-mutants and to measure

endocannabinoid content in several brain areas of the animals, in an attempt to find putative mechanisms through which the cross-talk between cannabinoid and NO systems are interacting. Another interesting consequence of the results obtained in Chapter 2 is the putative existence of a functional cross-talk between the cannabinoid system and cholecystokinin (CCK). As discussed in this Chapter, CB1 and CCK often have counteracting effects. Our group has recently obtained from Prof Linda Samuelson (U.S.A.) mutant mice carrying a deleted CCK gene. In the near future, we will analyse the interactions between these two systems by treatment of this CCK-mutant with cannabinoids and by treatment of null (CBN) or "conditional" CB1-mutants with CCK receptor agonists. Moreover, we will also analyse the expression pattern of genes involved in the cannabinoid and cholecystokinergic systems in these mouse strains, respectively.

The work described in Chapter 4 has occupied my time during the entire duration of my thesis and is still ongoing. In this Chapter, a "conditional" gene targeting approach is described. Two mouse lines were obtained: "CBN", carrying a null mutation of CB1, and "Floxed CB1", representing the key element for a future spatio-temporal restricted deletion of CB1. One aspect of the phenotype of the first line is already described in this Thesis (Chapter 5), but other experiments are also ongoing in our lab and in our Institute. For example, electrophysiological experiments revealed that induced long term potentiation is improved in the amygdala of homozygous CBN/CBN mice (Marsicano, Rammes & Lutz, unpublished results). This is a very interesting aspect that could indicate a central role of CB1 in amygdala-dependent forms of learning and memory, such as cued fear conditioning. Experiments in this direction are planned in our laboratory. The "Floxed CB1" mouse line will be the central tool for the "conditional" deletion of the gene. As discussed in Chapter 4, the coexpression data obtained in Chapter 2 represent very interesting findings also in view of a spatio-temporal specific

deletion of CB1. In fact, the regulatory sequences of CCK and glutamic acid decarboxylase (GAD) might provide a specific spatial restriction to the expression of Cre recombinase in transgenic mice. Together with the use of drug-inducible forms of Cre (see Chapter 4, 4.4 "Discussion"), it will be possible to obtain a very finely regulated deletion of the CB1 in cholecystokinergic and GABAergic neurons, respectively. In collaboration with other members of Dr Lutz's laboratory, the generation of these specific Cre-expressing transgenic mouse lines is planned for the near future.

In Chapter 5, neuroprotective effects of cannabinoids were analysed *in vitro*. The involvement of CB1 in these cannabinoid-mediated effects was analysed by heterologous expression in neuronal cell lines and in primary neuronal cultures derived from wild type and homozygous CBN/CBN mice. The results would indicate that CB1 is not necessary for cannabinoid-mediated *in vitro* neuroprotection in oxidative stress assays, as protection appears to be principally due to chemical antioxidant properties of cannabinoids. However, a non-antioxidant CB1 agonist (WIN 55,212-2) seems to exert CB1-independent neuroprotective effects. Also this aspect of the cannabinoid system will be further studied in the future, with the aid of CB1-mutant animals. As an example, it will be very interesting to analyse the differential responses of wild type and mutant ("null" or "conditional") to various neurotoxic conditions *in vivo*.

In conclusion, the preparation of this thesis represented a very exciting and challenging set of tasks, which allowed me to acquire knowledge of several technical and theoretical approaches to neurobiology and to be bewitched by the fascinating world of the cannabinoid system. For these reasons, I wish to keep on working on this specialized but apparently central aspect of mammalian brain physiology in the future.

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